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<b>Title</b>	Orally administered CLA ameliorates DSS-induced colitis in mice via intestinal barrier improvement, oxidative stress reduction, inflammatory cytokine and gut microbiota modulation
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<b>Publication date</b>	2019-11-06
<b>Original citation</b>	Chen, Y., Yang, B., Ross, R. P., Jin, Y., Stanton, C., Zhao, J., Zhang, H. and Chen, W. (2019) 'Orally administered CLA ameliorates DSS-induced colitis in mice via intestinal barrier improvement, oxidative stress reduction, inflammatory cytokine and gut microbiota modulation', Journal of Agricultural and Food Chemistry, 67(48), pp. 13282-13298. doi: 10.1021/acs.jafc.9b05744
<b>Type of publication</b>	Article (peer-reviewed)
<b>Link to publisher's version</b>	<a href="http://dx.doi.org/10.1021/acs.jafc.9b05744">http://dx.doi.org/10.1021/acs.jafc.9b05744</a> Access to the full text of the published version may require a subscription.
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<b>Embargo information</b>	Access to this article is restricted until 12 months after publication by request of the publisher.
<b>Embargo lift date</b>	2020-11-06
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Bioactive Constituents, Metabolites, and Functions

**Orally Administered CLA Ameliorates DSS-induced Colitis  
in Mice via Intestinal Barrier Improvement, Oxidative Stress  
Reduction, Inflammatory Cytokine and Gut Microbiota Modulation**

Yang Chen, Bo Yang, R. Paul Ross, Yan Jin, Catherine Stanton, Jianxin Zhao, Hao Zhang, and Wei Chen

*J. Agric. Food Chem.*, **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.9b05744 • Publication Date (Web): 06 Nov 2019

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4    Yang Chen<sup>†, ‡</sup>, Bo Yang<sup>†, ‡, ζ, \*</sup>, R. Paul Ross<sup>ζ, ξ</sup>, Yan Jin<sup>ψ</sup>, Catherine Stanton<sup>ζ, θ, ξ, \*</sup>, Jianxin  
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## ABSTRACT

Dietary supplementation with CLA has been reported to alleviate the effect of colitis in mice, but the mechanisms involved need further exploration. The study aimed to investigate how orally administered CLA alleviates DSS-induced colitis in mice. CLA was administered at five different doses: 40 mg/d, 20 mg/d, 10 mg/d, 5 mg/d and 2.5 mg/d. Doses of CLA at 10 mg/d and higher alleviated colitis symptoms and reduced inflammation induced by DSS, in which 40 mg/d, 20 mg/d and 10 mg/d CLA significantly increased the concentration of MUC2 and goblet cells, but neither 5 mg/d CLA nor 2.5 mg/d CLA had any effects. Meanwhile, 40 mg/d CLA and 20 mg/d CLA treatments significantly up-regulated the concentration of tight junction proteins (ZO-1, occludin and claudin-3) and ameliorated epithelial apoptosis caused by DSS. Moreover, oxidative stress-related enzymes (SOD, GSH-PX, CAT) and inflammatory cytokines (TNF- $\alpha$ , IL-10, IL-6) were modulated by 40 mg/d CLA and 20 mg/d CLA. Furthermore, 40 mg/d CLA rebalanced the gut microbiota damaged by DSS, including reducing *Bacteroides* and increasing *Bifidobacterium* and *Odoribacter*. In conclusion, CLA supplementation alleviated DSS-induced colitis in a dose-dependent manner by modulating inflammatory cytokines and oxidation stress, maintaining the mucosal barrier and reverting microbiota changes.



**KEYWORDS:** conjugated linoleic acid, colitis, intestinal barrier function, oxidative stress, gut microbiota

## INTRODUCTION

Conjugated linoleic acid (CLA) was the positional and geometric isomers of linoleic acid. Twenty eight CLA isomers have been identified from milk, dairy and ruminant meat.<sup>1</sup> The predominant isomer in dietary sources is cis9, trans11-CLA (c9, t11-CLA) which constitutes up to 90% of total CLA<sup>2</sup> and is associated with positive health benefits.<sup>3-5</sup> Trans10, cis12-CLA (t10, c12-CLA) is another common isomer which accounts for 1-10% of total CLA from diet<sup>6</sup> and is associated with anti-obesity effects.<sup>7-9</sup> CLA has demonstrated potent immunomodulatory effects that are exhibited in an isomer specific manner. These effects have been demonstrated in a wide range of inflammatory based disorders including inflammatory bowel disease (IBD),<sup>10, 11</sup> atherosclerosis<sup>12-14</sup> and diabete.<sup>15-18</sup>

IBD comprises Crohn's disease (CD) and ulcerative colitis (UC); their main characteristic is intestinal mucosal inflammation, and patients may have frequent recurrences and severe clinical forms.<sup>19-21</sup> Though the etiology of IBD is not fully understood, several factors, including intestinal barrier dysfunction, immunologic abnormalities, expansion of inflammatory mediators and oxidative stress are involved in the pathogenesis of IBD.<sup>22, 23</sup> 5-aminosalicylic acid (5-ASA), corticosteroids, particularly prednisone, hydrocortisone, and budesonide, have yielded positive

results in IBD treatment by inhibiting inflammation.<sup>24, 25</sup> However, prolonged use of this type of drug may result in other diseases such as hypertension, diabetes and osteoporosis.<sup>22, 25, 26</sup> New therapies for IBD which differ from traditional pharmacological treatments are being investigated and include prebiotics and some microbial metabolites such as unsaturated fatty acids.<sup>27, 28</sup>

It is worth noting that CLA has been shown to relieve IBD symptoms in animal models.<sup>10-11</sup> Feeding DSS-challenged C57BL/6J mice and their PPAR $\gamma$ -knock-out derivatives 1% CLA-supplemented diets proved that CLA was able to reduce colitis by activating PPAR- $\gamma$ .<sup>29</sup> In C57BL/6J colitis mice, CLA supplementation (100 mg/kg/day) prevented colonic shortening, significantly reduced the disease activity index and NF- $\kappa$ B expression, and caused an increase of PPAR- $\gamma$  and trefoil factor family 3 (TFF3) expression.<sup>30</sup> Evans et al.,<sup>31</sup> found that administration of a CLA-supplemented diet (1 g CLA/100 g diet) to C57BL/6J colitis mice improved disease activity, decreased expression of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and prevented colitis in part through a PPAR- $\gamma$ -dependent mechanism. Furthermore, a diet supplemented with 1% CLA in C57BL/6J colitis mice reduced mucosal damage and inflammatory mediator infiltration suggesting a PPAR- $\gamma$ -dependent mechanism mediated by macrophages.<sup>32</sup> These studies differed in terms of CLA concentrations administered to mice. Furthermore, the exact amount of CLA ingested by the mice and the concentration of CLA entering the intestinal tract of mice remains unclear. Moreover, it is important to fully comprehend the mechanisms by which CLA relieves colitis.

Against this background, the aim of the current study were to identify biologically

effective concentrations of orally administered CLA in mice and to uncover new potential pathways by which CLA alleviates colitis. The results from this study elucidate the relationships between the oral dose of CLA, the CLA content reaching the colon and colitis remission effect and provides more mechanisms by which CLA relieves colitis.

## **MATERIALS AND METHODS**

### **Animals and Experimental Design**

Male C57BL6/J mice (n = 64), 8-week-old and weighing 22-24 g, were raised at room temperature (25°C±2°C) and photoperiod (12 h/12 h light/dark period) in the barrier facility of Animal Center of Jiangnan University. Then, the 64 mice were divided into 8 groups (n=8/group) and fed standard chow and sterile water.

Table 1 showed the experimental procedures. Briefly, 2.5% (w/v) DSS (molecular weight 36,000-50,000, MP Biomedicals, LLC, Irvine, CA, USA) was added to the drinking water to induce colitis; the control group was fed with 200 µL skim milk (13% w/v) daily; the medically treated group, termed mesalazine group received 200 µL 10 mg/mL mesalazine (Etiasa pharmaceutical Co., Ltd., Saint-Cloud, Paris, France), while five CLA groups orally received 200 µL of different concentrations of CLA (emulsified with 13% w/v skim milk). CLA (50:50 mixture of c9, t11 and t10, c12 CLA isomers, purity: >99%, Nu-Check-Prep, Elysian, MN, USA) was emulsified with 13% w/v skim milk at different concentrations. The orally administered CLA concentrations were 40, 20, 10, 5 and 2.5 mg/d, respectively. The protocol for present study was approved by

the Ethics Committee of Jiangnan University, China (JN.No20180615c0560730[109]) and complied with the Directive of 2010/63/ European Community.

#### **Assessment of Colitis**

During DSS treatment, the changes of body weight and the disease activity index (DAI) of mice were measured everyday as the method of previous literature.<sup>33, 34</sup> Colon length was measured after dissecting the mice. Colon tissues were collected, dehydrated, embedded, sliced, and stained with Haematoxylin and Eosin (H&E) as the previous method.<sup>35</sup> The valuation system of pathological score was referred to previous reported.<sup>36</sup>

#### **Biochemical Assays**

The freshly excised colon was rinsed, homogenized in tissue lysis buffer, and then centrifuged at  $10,000 \times g$  at 4 °C for 15 min. The change of myeloperoxidase (MPO) activity, cyclooxygenase 2 (COX-2) activity and inducible nitric oxide synthase (iNOS) activity in the colon were assessed by commercially available ELISA kits (Nanjing Senbeijia Biotechnology Co., Ltd., Nanjing, Jiangsu, China) according to the manufacturer's instructions. The change of the colonic malonic dialdehyde (MDA), superoxide dismutase (SOD) activity, catalase (CAT) activity and glutathione peroxidase (GSH-PX) activity were assessed by the corresponding Kit (Nanjing Jiancheng Co., Ltd., Nanjing, Jiangsu, China). The protein concentration was measured by the BCA method using the BCA Protein Assay Kit (Beyotime Biotechnology,

Shanghai, China). The activities of MPO, COX-2, iNOS in the colon were presented as pictograms U/g colon protein, while that of SOD, CAT and GSH-PX were presented as pictograms U/mg colon protein.

### **Alcian Blue and Periodic Acid-schiff (PAS) Staining**

Distribution of mucin in the colon was investigated by alcian blue staining as the method of Steedman.<sup>37</sup> The number of goblet cells was investigated by PAS staining as previously described.<sup>38</sup>

### **The Level of Cytokines in Colon Tissue**

The concentrations of IL-4, TNF- $\alpha$ , IL-1 $\beta$ , IL-10 and IL-17 were measured by commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) in the supernatants of freshly isolated pieces of colon tissue homogenized with potassium-phosphate buffer (1% protease inhibitor cocktail) and centrifuged at  $10,000 \times g$  at 4 °C for 15 min. The protein concentration was measured by the BCA method using the BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) and the results were expressed as pg/mg protein of colon.

### **Measurement of Tight Junction Proteins**

The concentrations of E-cadherin 1, occludin, ZO-1 and claudin-3 were measured by commercially available ELISA kits (Nanjing Senbeijia Biotechnology Co., Ltd., Nanjing, Jiangsu, China) in the supernatants of freshly isolated pieces of colon tissue

homogenized with potassium-phosphate buffer (1% protease inhibitor cocktail) and centrifuged at  $10,000 \times g$  at 4 °C for 15 min. The protein concentration was measured by the BCA method using the BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) and the results were expressed as pg/mg protein of colon.

#### **Fatty Acid Analysis**

The extraction and methylation of fatty acid in blood, liver and colon were performed as previous described.<sup>35, 39</sup> Then, fatty acid were recovered with hexane and measured by GC-MS (the parameters of the instrument was described as previously described).<sup>35</sup> The temperature programming of the gas chromatography was described as the method of Yang et al.<sup>40</sup>

#### **Measurement of Transmission Electron Microscopy (TEM)**

The integrity of the tight junction (TJ) proteins of epithelial cells was assessed by measuring TEM. Colon tissues were collected, immobilized, dehydrated, embedded, sliced, and stained as the previous method.<sup>41</sup> Then, the sections were detected under HITACHI H8100 TEM (Hitachi, Tokyo, Japan) with an accelerating voltage of 200 kV.

#### **DNA Extraction, PCR Amplification, Sequencing and Bioinformatics Analysis**

Faecal samples from mice were collected to assess the changes in the composition of intestinal flora. Metagenomic DNA of the fecal samples was extracted by using a FastDNA Spin Kit for Feces (MP Biomedicals, LLC, Irvine, CA, USA). The V3-V4

region of the 16S rRNA gene was PCR amplified using primers (341F: 5'-CCTAYGGGRBGCASCAG-3'; 806R: 5'-GGACTACNNGGGTATCTAAT-3') as the method of Yang. et al.<sup>35</sup> After sequencing, bioinformatics analysis of the 16S rRNA sequence data was conducted as previously described.<sup>42</sup>

## Statistical Analysis

GraphPad Prism 7 and SPSS 22.0 were used to analyze data. P value of < 0.05 was considered to indicate statistical significance. Microbiota-related analyses were conducted by QIIME and R 3.5.0. Linear Discriminant Analysis Effect Size (LEfSe) were performed by python 2.7 and R 3.5.0. Network diagram of sample and OTU and bacterial-interaction patterns of the validation cohort were performed by Cytoscape 3.6.0.

## RESULTS

### CLA Improved the Colitis Symptoms

The changes of body weight and DAI were measured daily during DSS treatment. Body weight of mice dropped significantly (Figure 1A) while the DAI rose continuously (Figure 1B) due to DSS treatment. DSS treatment resulted in 12.3% weight loss at the end of the trial compared with the animals' initial weight (Figure 1A). However, the body weight of the mice in control group showed no significant difference. Compared with the DSS group, the weight loss of all the five CLA groups showed no significant difference.

Treatment with different concentrations of CLA had different effects on DAI. The DAI increased to  $10.50 \pm 0.38$  in the DSS group. The alleviating effects of mesalazine (DAI= $7.13 \pm 0.34$ ,  $P < 0.01$ ), 40 mg/d CLA ( $6.13 \pm 0.61$ ,  $P < 0.01$ ), 20 mg/d CLA ( $7.12 \pm 0.64$ ,  $P < 0.01$ ) and 10 mg/d CLA ( $7.37 \pm 0.72$ ,  $P < 0.05$ ) on colitis were significant when compared with the DAI of the DSS group (Figure 1B). 5 mg/d CLA and 2.5 mg/d CLA treatment led to an insignificant decrease in the DAI ( $8.87 \pm 0.48$ ,  $9.25 \pm 0.31$ ) compared with the DSS treatment.

The colon length in the control group was  $7.11 \pm 0.18$  cm (Figure 1C). The colons was normal reddish and the feces was granular in the control group. In comparison, the colon length of DSS-treated mice was  $5.8 \pm 0.12$  cm, which showed dark red colons, swollen, bleeding intestinal wall (Figure 1D). DSS treatment led to a 16.4% reduction of colon length compared with the control group, whereas mesalazine, 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA treatments prevented the colon shortening process by 5.6%, 5.4%, 5.5% and 9.5%, respectively (Figure 1C). Thus, mesalazine, 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA treatment could significantly prevent colon shortening, but 5 mg/d CLA and 2.5 mg/d CLA could not, consistent with the results of DAI.

### **CLA Recovered the Damage in Colonic Tissue Caused by DSS and Regulated Inflammatory Enzymes**

H&E staining was used to evaluate the histopathological injury. The colons of normal mice had intact mucous membranes and neat villi with healthy crypt structure (Figure 2A). The colon tissue of normal mice was enriched in goblet cells without inflammatory



cell infiltration or mucosal erosion. However, the mice in the DSS group showed intestinal mucosa and submucosal edema, severe inflammatory cell infiltration, crypt loss and epithelial injury.

The colon injury score of the DSS-treated mice ( $13.75 \pm 0.25$ ) was significantly higher than that of the normal mice ( $1.38 \pm 0.38$ ) ( $P < 0.0001$ ) (Figure 2B). 40 mg/d CLA and mesalazine treatment significantly improved the inflammation of colon, and tissue damage was reduced to different extents in the 20 mg/d CLA and 10 mg/d CLA groups. Among all the groups, 40 mg/d CLA and mesalazine treatment showed more effects of protecting colon: the crypts were intact and no significant disappearance for the goblet cells. Furthermore, 40 mg/d CLA and mesalazine treatment showed the least edema, and the least extent of inflammatory cell infiltration in the submucosa and serosa. The colon tissue injury scores of the mice treated with 40 mg/d CLA and mesalazine were  $3.13 \pm 0.29$  and  $4.00 \pm 0.42$ , respectively, similar to that of the control group. Fossae deformation, partial loss of mucosal epithelial cells, structural damage of the muscular layer were found in the mice of 20 mg/d CLA and 10 mg/d CLA groups. However, 5 mg/d CLA and 2.5 mg/d CLA treatments did not protect against the colon tissue damage. The tissue injury score in 5 mg/d CLA and 2.5 mg/d CLA was  $12.25 \pm 0.45$  and  $13.38 \pm 0.38$ , close to DSS group.

In order to evaluate the effect of CLA on colonic inflammatory enzymes, MPO, COX-2, and iNOS activities were measured. Mesalazine, 40 mg/d CLA, 20 mg/d CLA, 10 mg/d CLA, 5 mg/d CLA and 2.5 mg/d CLA treatment decreased the MPO activity induced by DSS from  $4.83 \pm 0.21$  to  $3.26 \pm 0.16$ ,  $3.48 \pm 0.26$ ,  $3.66 \pm 0.29$ ,  $3.41 \pm 0.54$ ,

4.05  $\pm$  0.27 and 3.99  $\pm$  0.32 U/g, respectively (Figure 2C). Apart from 5 mg/d CLA and 2.5 mg/d CLA groups, the MPO activity of mice in the other groups all showed significant differences when compared with the DSS group, which was consistent with the results of DAI and colon length. The DSS treatment resulted in highest COX-2 activity (27.25  $\pm$  1.934 U/g protein), while mesalazine, 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA treatment significantly decreased COX-2 activity (22.58  $\pm$  1.194, 22.65  $\pm$  1.039, 22.41  $\pm$  0.964 U/g protein) (Figure 2D). Moreover, the iNOS activities of the mice of all the seven groups were lower than that of the DSS group, although the differences were not significant (Figure 2E).

### **CLA Protected the Intestinal Barrier**

To evaluate the influence of CLA on the mucous layer and goblet cells, the concentration of mucin2 (MUC2) and goblet cell numbers were measured. The results of alcian blue and PAS staining showed that the goblet cells were severely damaged, and large amounts of MUC2 disappeared in DSS group (Figure 3A and 3B). Mesalazine, 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA treatments significantly protected the reduction of goblet cells and the destruction of mucosal layer.

The content of MUC2 was analyzed by ELISA. The concentration of MUC2 was significantly reduced in the mice of DSS treatment (66.74  $\pm$  1.52 pg/mg protein), whereas 40 mg/d CLA and mesalazine treatment could maintain its content (80.53  $\pm$  3.783 and 78.50  $\pm$  1.946 pg/mg protein, respectively) at normal levels compared with control (79.98  $\pm$  1.48 pg/mg protein) (Figure 3C). Moreover, 20 mg/d CLA and 10 mg/d

265 CLA treatments significantly increased the content of MUC2 compared with the DSS  
266 group, while MUC2 in the 5 mg/d CLA and 2.5 mg/d CLA groups were similar to that  
267 of the DSS group. Remarkably, mice challenged with DSS suffered a loss of mucus-  
268 producing goblet cells compared with untreated mice. However, 40 mg/d CLA, 20 mg/d  
269 CLA and 10 mg/d CLA treatments significantly relieved the loss of goblet cells at a  
270 reasonable level ( $p < 0.01$ ) (Figure 3D). The concentration of MUC2 verified the above  
271 mentioned phenomenon (Figure 3C and 3D).

272 To evaluate the effect of CLA on the epithelium structure, TJ proteins and epithelial  
273 apoptosis were measured. Mesalazine, 40 mg/d CLA, 20 mg/d CLA, 10 mg/d CLA and  
274 5 mg/d CLA treatments increased the concentrations of ZO-1 by 20.7%, 50.7%, 51.9%,  
275 33.6% and 33.2%, respectively, compared with DSS treatment (Figure 4A). Moreover,  
276 DSS treatment decreased the concentration of E-cadherin 1, occludin and claudin-3  
277 compared with the control (Figure 4B, 4C, 4D). By contrast, 40 mg/d CLA and 20 mg/d  
278 CLA significantly increased the concentration of occludin and claudin-3. The  
279 concentrations of occludin and claudin-3 were up-regulated to certain levels in 10 mg/d  
280 CLA, 5 mg/d CLA and 2.5 mg/d CLA groups, but were not significantly different to  
281 DSS (Figure 4B and 4C). However, E-Cadherin1 levels in all the CLA groups were  
282 significantly higher than that in the DSS group (Figure 4D).

283 To further verify that CLA can affect the tight junction of intestinal epithelial tissues,  
284 TEM was used to observe the tight junction of intestinal epithelial tissues. In the control  
285 mice, intestinal barrier was intact, the microvilli (Mv) of the epithelial cells were neatly  
286 arranged, and TJ, adheres junction (AJ), desmosome (De) were integrate. However,

fractured a widened or fractured TJ, AJ and De, curved microvilli was observed in the DSS treated mice. These were improved significantly following 40 mg/d CLA treatment (Figure 4E), which showed that CLA can improve the TJ and AJ of intestinal epithelial tissues.

Furthermore, Hoechst 33258, a special fluorescent dye, was used to stain for the colon tissues. Hoechst 33258 could differentiate between apoptotic and normal cells by using a fluorescence microscope. The nuclei of normal cells show diffuse homogeneous blue fluorescence; however, apoptotic cells present with strong blue fluorescence. As expected, the control treatment did not induce apoptosis of cells, but when treated with DSS, typical morphological changes were observed, as the image displays, with nuclear fragmentation, chromosomal condensation and cell shrinkage (Figure 4F). 40 mg/d CLA, 20 mg/d CLA and mesalazine treatments could prevent apoptotic cells, while a large number of apoptotic cells appeared in the mice in 5 mg/d CLA and 2.5 mg/d CLA groups (Figure 4F).

### **CLA Regulated Oxidative Stress**

In order to evaluate the influence of CLA on oxidative stress, MDA level, CAT activity, GSH-PX activity and SOD activity of colon were measured. 40 mg/d CLA and 20 mg/d CLA treatment significantly increased SOD activity with a 1.22- and 1.20-fold compared with DSS treatment, respectively (Figure 5A). However, there was no significant difference among all the CLA groups and other groups for MDA (Figure 5B). In addition, mesalazine, 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA treatments

exhibited significant increments on GSH-PX activity (17.5%, 25.5%, 24.3% and 18.7%) ( $p < 0.05$ ) compared with DSS treatment (Figure 5C). DSS and 20 mg/d CLA treatment showed the lowest ( $35.65 \pm 1.11$  U/mg protein) and the highest ( $45.1 \pm 2.12$  U/mg protein) CAT activity, respectively. Furthermore, mesalazine, 40 mg/d CLA, 10 mg/d CLA and 2.5 mg/d CLA treatments showed significant increments on CAT activity compared with DSS treatment ( $p < 0.05$ ) (Figure 5D).

### **CLA Regulated Inflammatory Cytokines**

In order to evaluate the influence of CLA on inflammatory factors, TNF- $\alpha$ , IL-1 $\beta$ , IL-10 and IL-6 concentrations were analyzed by ELISA. Inflammatory factors in the colon of DSS-treated mice were significantly higher than those in the normal mice, with significant increases in TNF- $\alpha$ , IL-1 $\beta$  and IL-6 (Figure 6). Notably, IL-1 $\beta$ , the most significant pro-inflammatory cytokine, increased by 2.42-fold in the colon (Figure 6A). CLA-feeding decreased the concentrations of colonic TNF- $\alpha$  and IL-6 (Figure 6B and 6C). Compared with the DSS treatment group, 40 mg/d CLA and 20 mg/d CLA treatment significantly decreased the concentration of TNF- $\alpha$  in the colon (Figure 6B). In mesalazine, 40 mg/d CLA and 20 mg/d CLA groups, the concentration of IL-6 in the colon was significantly lower than in the DSS group; however, no significant differences were observed for 10 mg/d CLA, 5 mg/d CLA, 2.5 mg/d CLA and DSS groups (Figure 6C). Mesalazine treatment significantly decreased the concentration of IL-1 $\beta$ , while the mice of all the CLA groups showed no significant reduction compared to that in the DSS group (Figure 6A). Moreover, the concentration of the anti-

inflammatory cytokine, IL-10, in mesalazine, 40 mg/d CLA and 20 mg/d CLA groups increased to 52.7%, 57.1% and 33.6% compared with DSS group ( $35.26 \pm 0.93$  pg/mg protein), respectively (Figure 6D). Interestingly, 40 mg/d CLA, 20 mg/d CLA, 10 mg/d CLA and 5 mg/d CLA treatment significantly increased PPAR- $\gamma$  concentration, while mesalazine treatment did not (Figure 6E). Overall, 40 mg/d CLA exerted the most significant inflammatory modulation effect, followed by mesalazine and 20 mg/d CLA.

### **Correlation between Tight Junction Proteins, Antioxidant Enzymes and Cytokines Regulated by CLA and Colitis Indices in Mice**

In order to evaluate the relationship between tight junction proteins, antioxidant enzymes and cytokines regulated by CLA and colitis indices, the spearman correlation was analyzed. Different concentrations of CLA had different effects on the content of tight junction proteins, antioxidant enzymes and cytokines in mice colon tissue. The concentrations of ZO-1, occludin and E-Cadherin1 showed extremely negative correlations with DAI and tissue histological scores, while claudin-3 had no significant correlation with the colon length, MPO, DAI and tissue histological score (Figure 7A). Moreover, ZO-1 showed highly positive correlation with colon length, while occludin showed highly negative correlation with MPO.

In addition, the activity of GSH-PX and CAT displayed a high negative correlation with DAI and tissue histological scores. However, the concentration of MDA was the positive correlation with histological score and DAI. TNF- $\alpha$  and IL-6 showed high degree correlation with DAI and histological scores, but did not display correlation with

the colitis index MPO. Furthermore, IL-10 only showed negative correlation with histological score. IL-1 $\beta$  did not display correlation with any of the four colitis indices because CLA did not regulate it. It is notable that PPAR- $\gamma$  displayed a high degree of correlation with colon length, DAI and tissue histological scores (Figure 7A).

### **The Effect of Orally Administered CLA on the Concentration of CLA in the Colon, Blood and Liver**

In order to evaluate the distribution of orally administered CLA in mice, the CLA levels in colonic contents, blood and liver were analyzed. The concentrations of CLA in colonic contents from 40 mg/d CLA, 20 mg/d CLA, 10 mg/d CLA, 5 mg/d CLA and 2.5 mg/d CLA groups were  $1.347 \pm 0.153$ ,  $0.743 \pm 0.057$ ,  $0.614 \pm 0.11$ ,  $0.443 \pm 0.076$ ,  $0.226 \pm 0.092$  g/100g FAME, respectively, which were higher than that in the control group as well as mesalazine and DSS groups (Figure 7B). Similar to that observed in the colon, CLA concentrations in blood and liver from the CLA groups showed a decreasing trend from 40 mg/d CLA to 2.5 mg/d CLA in line with the decreasing concentrations of CLA administered to the mice (Figure 7C and 7D). The concentrations of CLA in colonic, blood and liver samples from control, mesalazine and DSS treatment groups were not statistically different from each other (Figure 7B, 7C and 7D).

In order to evaluate the effect of the concentration of CLA in the colon on inflammatory markers of colitis, the interdependent quantitative relationships between the colonic CLA concentration and inflammatory markers of colitis were analyzed via

unary linear regression. The concentration of colonic CLA displayed extremely negative correlations with DAI and tissue histological scores ( $p < 0.0001$ ). Moreover, colonic CLA showed a high positive correlation with colon length, while a highly negative correlation correlated with MPO ( $p < 0.05$ ). The results show that the amount of CLA reaching the colon directly correlated with the oral dose of CLA, and the content of CLA in the colon significantly and positively correlated with the relief effect of colitis.

### **Modulation of Intestinal Microbiota by CLA**

In order to evaluate the effect of CLA on the intestinal microbiota, the gut microbiota of mice treated with CLA at 40 mg/d, mesalazine group, DSS and control groups were investigated based on 16S rRNA-amplicon sequencing. Mice with chronic stress (DSS group) showed dramatic alteration of the gut microbial structure compared with the control. In the control group, the dominant phyla were Actinobacteria (19.27%), Bacteroidetes (32.16%), Firmicutes (36.41%) and Verrucomicrobia (5.75%) (Figure 8A). However, DSS treatment significantly changed the composition of bacteria at the phylum level, and the relative abundance of Bacteroidetes increased to 68.83% and the relative abundances of Actinobacteria, Firmicutes and Verrucomicrobia decreased to 3.76%, 16.70% and 2.06%, respectively (Figure 8A). Even though the relative abundance of Bacteroidetes in the CLA and mesalazine groups was higher than that in the control group, it was significantly decreased compared with the DSS group. Moreover, CLA treatment significantly increased the relative abundance of Firmicutes,



397 compared with DSS treatment. Alpha diversity was evaluated by Chao1 and Shannon  
398 index. After CLA treatment, Shannon index increased and was significantly different  
399 compared with microbiota from the DSS group, but Chao1 index showed no statistical  
400 differences compared with DSS treatment (Figure 8B and 8C). Beta diversity was  
401 reflected by principal coordinates analysis (PCoA) of weighted UniFrac distance. The  
402 results showed that the gut microbiota of DSS treatment mice was significantly  
403 different from the mice in the control group, and administration of CLA could remit the  
404 shift of gut microbiota induced by DSS treatment (Figure 8D). OTUs of all four groups  
405 were evaluated to identify the unique and shared genus. Different groups had their own  
406 distinct OTUs which were not shared with native controls. There were six, eight, eight  
407 and twenty-five distinct OTUs in control, DSS, mesalazine and CLA groups, while  
408 others OTUs were shared among those groups (Figure 8E).

409 The gut microbiota diversity among different groups was analyzed by LEfSe (LDA  
410 Effect Size). The LDA score histogram was drawn to identify statistically significant  
411 biomarkers and reveal the dominant microorganisms in each group (Figure 9A and 9B).  
412 Dominant communities of five, seven taxa and six taxa were found in the DSS,  
413 mesalazine and CLA groups, respectively. Among them, *Bacteroides* and  
414 Bacteroidaceae were the dominant in the DSS group; S24\_7 and Verrucomicrobia were  
415 the dominant in the mesalazine group; while Proteobacteria, Odoribacteraceae and  
416 *Odoribacter* were the dominant microbes in the CLA group (Figure 9B). Relative  
417 abundance of selected taxa showed that the abundance of S24-7, *Bifidobacterium*,  
418 *Lactobacillus* and *Akkermansia* significantly decreased in DSS treatment mice, but the

419 abundance of *Bacteroides* significantly increased (Figure 9C). Compared with the DSS  
420 group, CLA treatment mice showed an increased abundance of *Bifidobacterium* and  
421 *Odoribacter* ( $p < 0.05$ ) and a significantly decreased abundance of *Bacteroides* (Figure  
422 9C).

423 The effect of CLA treatment on bacterial interaction patterns were further analyzed.  
424 In DSS group, Clostridiaceae (OTU82) and Peptostreptococcaceae (OTU101) were the  
425 core microbes. Clostridiaceae correlated positively with Coriobacteriaceae (OTU20),  
426 *Enterobacter* (OTU172), *Blautia* (OTU93), *Eubacterium* (OTU124) and  
427 *Paraprevotella* (OTU39) (Figure 9D). Moreover, Peptostreptococcaceae had a positive  
428 correlation with *Staphylococcus* (OTU58), *Clostridium* (OTU122) and *Trabulsiella*  
429 (OTU180). In CLA treatment group, it was found that Enterobacteriaceae (OTU170)  
430 and *Anaeroplasma* (OTU194) were the core microbe. Enterobacteriaceae showed a  
431 positive correlation with *Pseudomonas* (OTU190), but negative correlation with  
432 *Anaeroplasma* (OTU194), *Ruminococcus* (OTU110), *Oscillospira* (OTU191),  
433 *Bifidobacterium* (OTU19), which was also the core microbe (Figure 9D). Thus, CLA  
434 treatment changed the core gut microflora and their interaction pattern.

435 Furthermore, the correlations among colonic CLA concentration, TJ proteins,  
436 differential microorganisms and inflammation markers were analyzed. Colon length,  
437 histological score, MPO and DAI were the most important indicators for colitis, and  
438 showed the bigger weightiness in the network analysis (Figure 9E). Colonic CLA  
439 concentration positively correlated with colon length, IL-10 and TJ proteins (occluding,  
440 ZO-1 and E-Cadherin1), in contrast, negatively correlated with *Bacteroides*,

histological score, DAI, MPO and pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ ). Interestingly, the relative abundance of *Bacteroides* were positively correlated with histological scores and DAI; however, it was negatively correlated with IL-10 and TJ proteins (occluding and ZO-1), indicating *Bacteroides* has a negative effect on colitis. It can be found that TJ proteins (occluding, ZO-1 and E-Cadherin1) had a negatively correlation with histological score, MPO and DAI. Furthermore, TNF- $\alpha$  and IL-10 showed an important correlation with the indicator of colitis. Notably, there was no significant correlation between the differential microorganisms and antioxidant related enzymes.

## DISCUSSION

Salicylate, steroids, immunosuppressants, and anti-tnf-alpha drugs are traditionally used to treat patients with IBD. However, different response rates and potential side effects appeared in these therapies. Thus, exploring novel therapeutic and preventive approaches for IBD is important and has attracted increasing interest.<sup>43-47</sup> A number of studies have demonstrated that CLA can ameliorate experimental IBD in mice and pigs.<sup>29-32, 48, 49</sup>

In this study, some clinical symptoms in mice were significantly alleviated by administering CLA to mice for 7 days prior to DSS treatment and continuing CLA administration for another 7 days simultaneously with DSS treatment. In particular, treatments 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA alleviated colon shortening, diarrhea and hematochezia. In addition, 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA

treatments significantly mitigated intestinal mucosa and submucosal edema, inflammatory cell infiltration, crypt loss and epithelial injury resulting from DSS challenge. Thus, 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA treatments significantly decreased DAI, histological injury score, and increased colon length, but 5 mg/d CLA and 2.5 mg/d CLA treatments did not elicit these effects, which indicates that CLA relieves colitis in a dose-dependent manner.

The mucosal barrier, the first line of protection of the intestinal tract, is mainly composed of mucous layer and epithelial cell layer. It prevents intestinal bacteria toxins and other exogenous substances from invading the intestinal tissues and, subsequently, prevents intestinal mucosal injury.<sup>50</sup> 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA treatments increased the concentration of MUC2, which could maintain the integrity of the colonic mucous layer and protect goblet cells. Moreover, the goblet cell numbers of the mice treated with 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA were higher than that of DSS treated mice, which was consistent with the results of MUC2. TJ proteins of enterocytes predominantly regulate the integrity of the intestinal barrier and play an important role in IBD.<sup>51</sup> In the present study, 40 mg/d CLA and 20 mg/d CLA treatment up-regulated TJ proteins (E-cadherin 1, ZO-1, claudin-3 and occluding). Thus, we conclude that to a certain extent high CLA concentrations can maintain the integrity of epithelium structure. A previous study by Wang and colleagues<sup>44</sup> showed that 1% CLA added in the diet up-regulated the mRNA levels of MUC2, E-cadherin 1, claudin-3, ZO-1 and occludin in DSS-induced colitis mice. Therefore, oral administration of CLA (40 or 20 mg/d) up-regulates the concentration of these TJ proteins, which indicates

that appropriate concentrations of CLA can improve the intestinal barrier function. Furthermore, 10 mg/d CLA and 5 mg/d CLA treatments increased the concentration of E-cadherin 1 and ZO-1 ( $p < 0.05$ ) compared with DSS treatment, while 2.5 mg/d CLA (2.5 mg/d) only increased the concentration of E-cadherin 1. Therefore, the regulation of CLA on TJ proteins occurred in a dose-dependent manner.

Oxidative stress can lead to abnormal oxygen free radical metabolism and excessive activation of apoptosis. Moreover, the oxygen free radicals lead to increase release of inflammatory mediators (such as cytokines and chemokines), which not only cause epithelial cell damage, but also aggravates oxidative stress, leading to the destruction of the intestinal mechanical barrier.<sup>52</sup> Chinnadurai and colleagues found that feeding high CLA (19.54 mg/g fat) enriched ghee to rats during the pubescent period resulted in an increase in CAT and SOD enzyme activities both in blood and liver.<sup>53</sup> Large yellow croaker fed with soybean oil-based diets plus 0.83% CLA significantly increased the activity of CAT and total antioxidant capacity (T-AOC) in liver.<sup>54</sup> Moreover, SOD, CAT and GSH-PX can ameliorate the peroxidation reactions in colitis.<sup>55</sup> In the current study, CLA (40 and 20 mg/d) significantly increased the activity of CAT, SOD and GSH-PX in colon tissue, which indicated that CLA alleviated colitis by inhibiting oxidative stress. Moreover, 10 mg/d CLA treatment increased the activities of CAT and GSH-PX in the colon, while only CAT activity was increased by 5 mg/d CLA and 2.5 mg/d CLA. Thus, CLA regulated oxidative stress related enzymes in a dose-dependent manner.

IBD may be associated with uncontrolled, highly activated inflammation of the

intestinal mucosa. Studies have shown that the anti-inflammatory effect of CLA was mainly achieved by regulating the expression and activity of PPAR $\gamma$ .<sup>56</sup> PPAR $\gamma$  is one of the three subtypes of PPARs ( PPAR $\alpha$ 、 PPAR $\beta/\delta$  and PPAR $\gamma$ ), in which PPAR- $\gamma$  belongs to the nuclear receptor superfamily.<sup>57</sup> PPAR $\gamma$  can inhibit the activation and nuclear import of NF- $\kappa$ B through the I $\kappa$ B- $\alpha$  pathway,<sup>58</sup> in which NF- $\kappa$ B plays a key role in the regulation of the inflammatory response and pathogenesis of IBD. Hontecillas et al.,<sup>48</sup> found that CLA can inhibit inflammation of the colon in a colitis model caused by pathogenic bacteria, and increase the expression of PPAR in the colon. Since then, Bassaganya-Riera et al.,<sup>29</sup> used PPAR $\gamma$  knockout mice to prove that CLA was able to reduce colitis by activating PPAR $\gamma$ . In colon cancer cell lines HT-29 and Caco-2, CLA induced cell apoptosis by up-regulation of PPAR $\gamma$ .<sup>59</sup> In the current study, 40 mg/d CLA, 20 mg/d CLA, 10 mg/d CLA, 5 mg/d CLA and mesalazine increased the concentration of PPAR $\gamma$ , which was consistent with previous research.

NF- $\kappa$ B pathway can be activated by TNF- $\alpha$ , which was produced by macrophages; at the same time, NF- $\kappa$ B could promote the secretion of TNF- $\alpha$ , IL-6 and IL-1 $\beta$ .<sup>60</sup> In RAW264.7, a macrophage cell-line from mice, CLA reduced the mRNA expression levels of INF- $\gamma$ , COX2, TNF- $\alpha$ , IL-1 and IL-6 genes through the PPAR $\gamma$  pathway.<sup>61</sup> Moreover, CLA significantly down-regulated the expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and up-regulated IL-10.<sup>44</sup> In our current study, CLA (40 or 20 mg/d) decreased the concentration of TNF- $\alpha$  and IL-6 while increasing that of IL-10 in the colon, which may be due to the activation of PPAR $\gamma$  and the inhibition of NF- $\kappa$ B, thus resulting in a lower secretion of pro-inflammatory cytokines (TNF- $\alpha$  and IL-6). However, these pro-

inflammatory cytokines were not up-regulated by 10 mg/d CLA, 5 mg/d CLA mg/d CLA treatments. Thus, inflammatory cytokines were regulated by CLA in a dose-dependent manner.

It is known for that in healthy individuals a symbiotic relationship exists between the gut microbiota and host, and the gut microbiota is closely related to the pathogenesis of IBD.<sup>62</sup> With PCoA analysis, it was found that the gut microbiota of DSS-treated mice was dramatically different from that of the control group, but CLA (40 mg/d) treatment mitigated gut microbiota shift induced by DSS challenge. At the phylum level, the relative abundance of Bacteroidetes in the DSS treatment group was 2.14 times more than that in the control group, while that in the CLA (40 mg/d) administered group significantly decreased compared with the DSS group. Moreover, CLA treatment increased the relative abundance of Verrucomicrobia, which was considered to be associated with the higher expression of MUC2.<sup>63</sup> At the genus level, CLA treatment increased the abundance of *Bifidobacterium* and *Odoribacter* and significantly decreased the abundance of *Bacteroides* compared with DSS treatment. *Bifidobacterium* was reported to remit colitis.<sup>64</sup> Moreover, *Odoribacter* was reduced as a result of DSS treatment, which could ameliorate ulcerative colitis, and may increase host inflammation by reducing production of short chain fatty acids.<sup>65</sup> Most commonly, *Odoribacter* is known for of its ability to produce butyrate,<sup>66</sup> which can improve the intestinal barrier and relieve colitis.<sup>67, 68</sup> Notably, the current results showed that *Odoribacter* had a positive correlation with occludin and negative correlation with IL-6. Some *Bacteroides fragilis* strains can invade intestinal tissue and cause damage.<sup>69</sup> In

addition, certain *B. vulgatus* and *B. ovatus* have been found to affect the development of IBD.<sup>70-72</sup> In the present study, the results showed that *Bacteroides* were negatively correlated with TJ proteins (occluding and ZO-1), IL-10 and positively correlated with inflammatory markers of colitis (DAI and histological score), which was consistent with previous results.<sup>70-72</sup>

Sokol et al.,<sup>73</sup> reported a skewed microbial interaction pattern in IBD patients and found that the concomitant analysis of microbiota showed a dense and homogenous correlation network in healthy subjects, but an unbalanced network in IBD patients. In the present study, unbalanced microbiota was found in DSS treatment group, in which the core microbes were Clostridiaceae, Peptostreptococcaceae, *Staphylococcus*, *Trabulsiella* and *Paraprevotella*, and correlated positively with Coriobacteriaceae, *Enterobacter*, *Eubacterium*. Additionally, Clostridiaceae, Peptostreptococcaceae, *Staphylococcus*, *Trabulsiella*, *Enterobacter* and *Paraprevotella* have been confirmed to aggravate colitis.<sup>74, 75</sup> However, CLA treatment could improve the unbalanced microbiota. In CLA treatment group, Enterobacteriaceae, one of the core microbes, showed a positive correlation with *Pseudomonas*, but negative correlation with *Anaeroplasma*, *Ruminococcus*, *Oscillospira* and *Bifidobacterium*. Interestingly, *Ruminococcus*, *Oscillospira* and *Bifidobacterium* were reported to improve colitis.<sup>64, 74, 75</sup> Meanwhile, *Oscillospira* could produce butyrate to improve the intestinal barrier and relieve colitis,<sup>67, 68, 74</sup> and *Ruminococcus* was negatively correlated with CD.<sup>75</sup> Thus, our results indicated that CLA (40 mg/d) treatment partially prevented the microbiota changes induced by DSS.



573 Thus, orally administrated CLA resulted in some CLA entering the colon, where it  
574 acts as an anti-inflammatory agent. Interestingly, the present study found a significant  
575 positive correlation between CLA content in the colon and the relief effect of enteritis.  
576 When the oral dose of CLA exceeded 10 mg/d, 0.613 mg/mL CLA reached the colon,  
577 which has a relief effect on colitis; however, when the oral dose of CLA was 5 mg/d,  
578 0.443 mg/mL CLA reached the colon, showing no significant improvement in colitis.  
579 This suggests that CLA does have a dose-dependent relationship in relieving colitis. In  
580 view of the efficacy of CLA for mice, clinical trials investigating the efficacy of CLA  
581 in UC patients need to be conducted in the future. According to the effective dose of  
582 the current study and the dose conversion relationship between animals and humans,  
583 the oral dose of CLA in future clinical trials of UC patients should be more than 42  
584 mg/kg body weight.

585 The primary mechanisms that CLA significantly ameliorated DSS-induced colitis  
586 involved in inhibiting pro-inflammatory factors, maintaining mucosal barriers,  
587 regulating oxidative stress and intestinal microbial damage. From all those results, it  
588 can found that CLA entered into the bowel lumen then decreased the abundance of  
589 *Bacteroides* and increased the abundance of *Bifidobacterium*, which could impact the  
590 concentration of AJ proteins and inflammatory cytokines. Furthermore, CLA that in the  
591 bowel lumen could improve antioxidant related enzymes, which could increase AJ  
592 proteins and improve intestinal barrier. Simultaneously, CLA could directly penetrate  
593 into the mucus layer and epithelial cells to regulate MUC2 and AJ proteins.  
594 Additionally, CLA could enter into the lamina propria in the mice treated DSS, then

reduced inflammation and regulated the cytokines. Thus, CLA could not only indirectly improve intestinal barrier and regulate inflammatory factors through the regulation of bacterial flora and oxidative stress, but also could directly regulate mucin and TJ protein as well as inflammatory factors, which could directly improve related indexes of colitis. These results will help us understand the mechanisms by which CLA alleviates colitis and regulates other immune-related diseases, and hence guide further development of CLA products.

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### **Funding**

This research was supported by the National Natural Science Foundation of China (Nos. 31801521, 31722041), the Fundamental Research Funds for the Central Universities (No. JUSRP51702A), National First-Class Discipline Program of Food Science and Technology (JUFSTR20180102), Postgraduate Research & Practice Innovation Program of Jiangsu Province (KYCX19\_1829), Wuxi Young Talent Foundation (QNRC075) and the Jiangsu Province “Collaborative Innovation Center for Food Safety and Quality Control”.

### **Notes**

The authors declare no competing financial interest.

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881     **Table 1** Animal model experimental design.

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Control	13% wt/v skim milk 200 µL once a day (day1-day14)							13% wt/v skim milk 200 µL once a day (day1-day14)						
DSS	13% wt/v skim milk 200 µL once a day (day1-day7)							2.5% (w/v) DSS in water+13% wt/v skim milk 200 µL once a day (day8-day14)						
Medicine	10 mg/mL mesalazine 200 µL once a day (day1-day7)							2.5% (w/v) DSS in water+10 mg/mL mesalazine 200 µL once a day (day8-day14)						
CLA-1	200 mg/mL CLA 200 µL once a day (day1-day7)							2.5% (w/v) DSS in water+200 mg/mL CLA 200 µL once a day (day8-day14)						
CLA-2	100 mg/mL CLA 200 µL once a day (day1-day7)							2.5% (w/v) DSS in water+100 mg/mL CLA 200 µL once a day (day8-day14)						
CLA-3	50 mg/mL CLA 200 µL once a day (day1-day7)							2.5% (w/v) DSS in water+50 mg/mL CLA 200 µL once a day (day8-day14)						
CLA-4	25 mg/mL CLA 200 µL once a day (day1-day7)							2.5% (w/v) DSS in water+25 mg/mL CLA 200 µL once a day (day8-day14)						
CLA-5	12.5 mg/mL CLA 200 µL once a day (day1-day7)							2.5% (w/v) DSS in water+12.5 mg/mL CLA 200 µL once a day (day8-day14)						

882

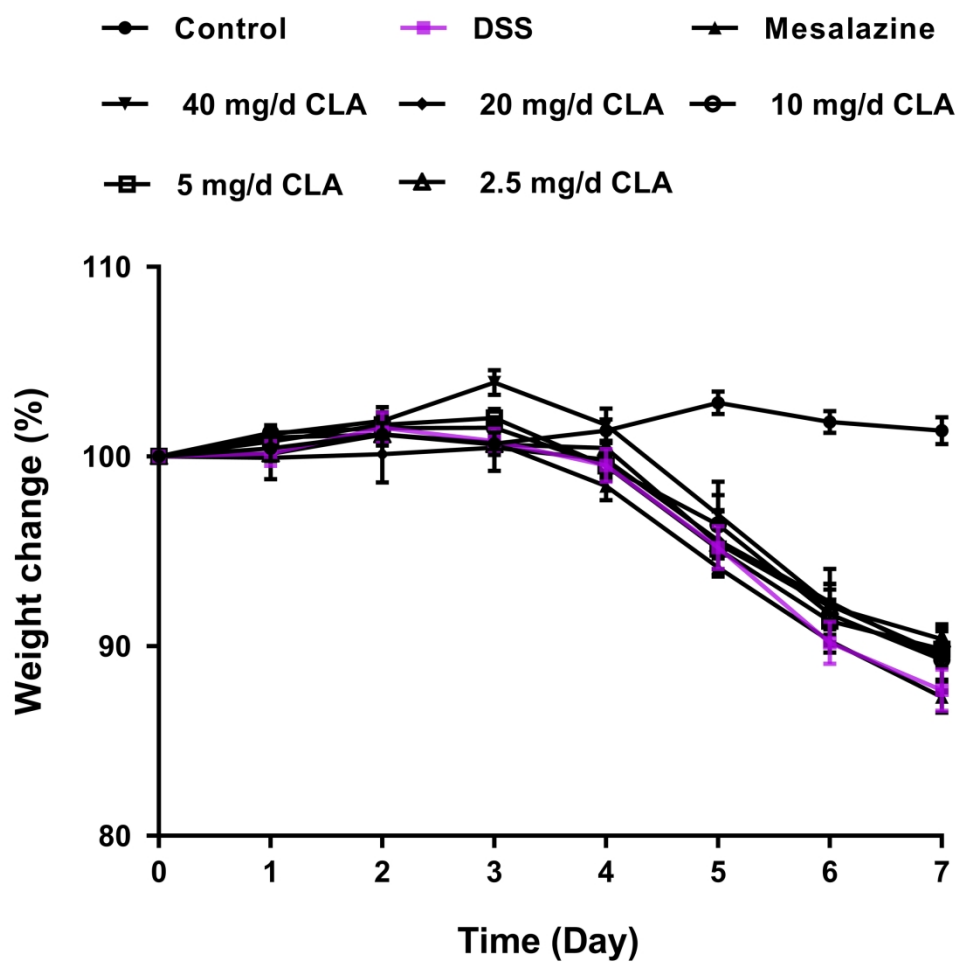
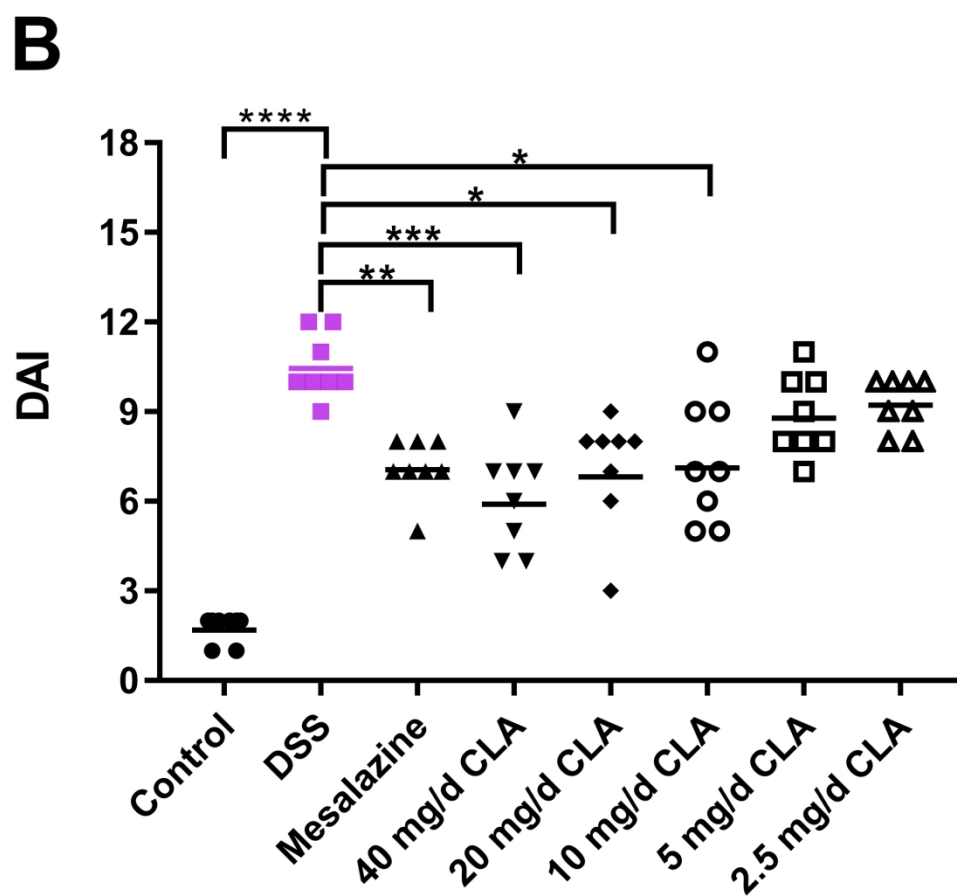
**A**

Fig.1 Symptoms of DSS-induced colitis. (A) Body weight, (B) Disease activity index (DAI), (C) Colon length, (D) Macroscopic pictures of colons. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ , \*\*\*\*:  $p < 0.0001$ . All data are presented as mean  $\pm$  SEM (n=8 mice per group).

234x245mm (300 x 300 DPI)





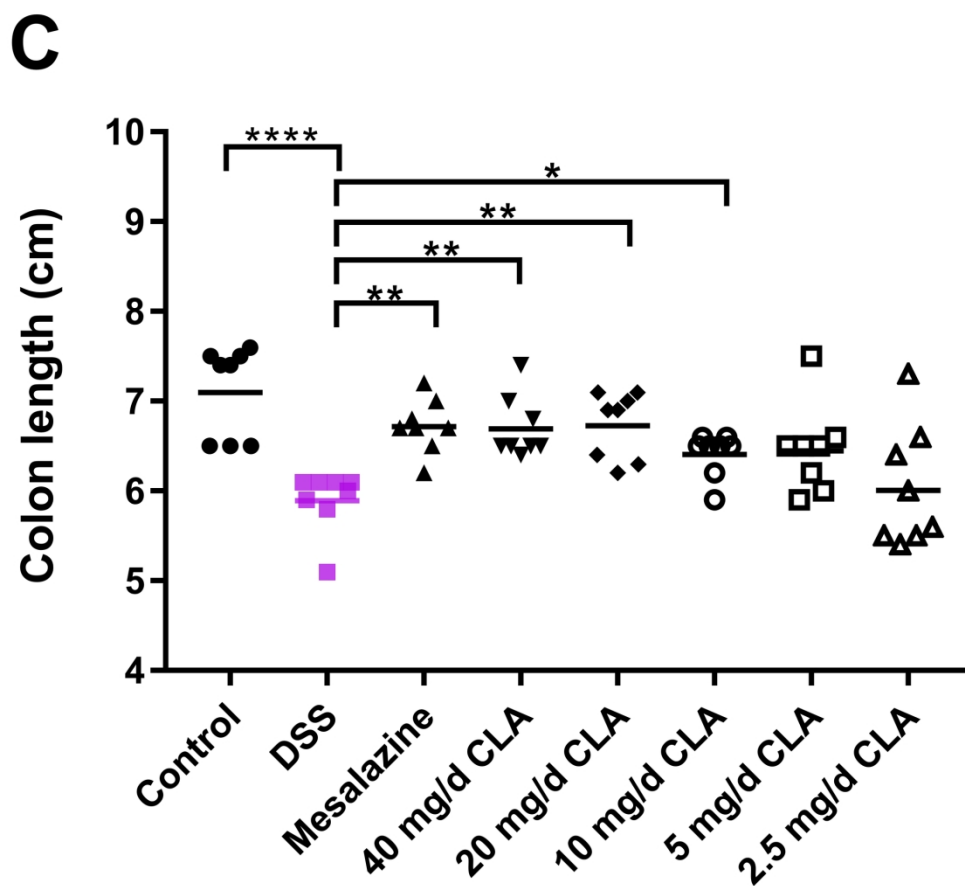


Fig.1C

188x169mm (300 x 300 DPI)

D

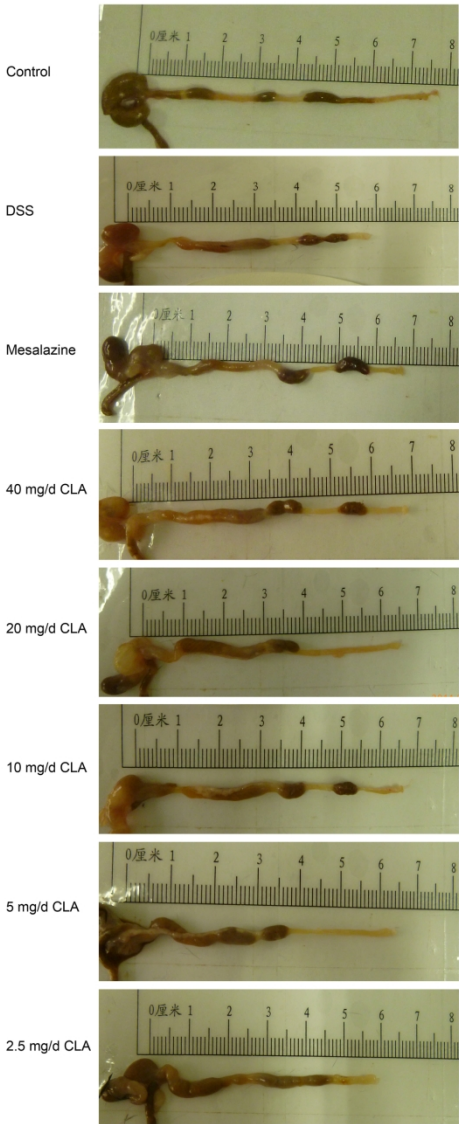


Fig.1D

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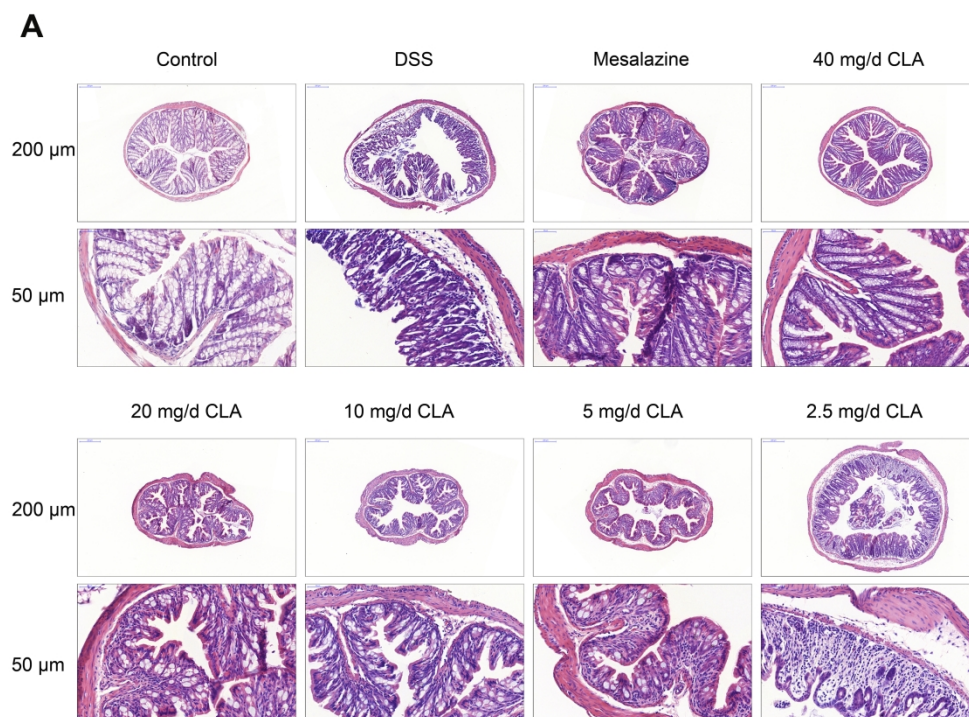


Fig.2 Effect of CLA on the histological injury and enzyme activities in colon of colitis. (A) Histological examination, Scale bars, 200 and 50  $\mu$ m, (B) Colonic histological injury, (C) MPO, (D) COX-2, (E) iNOS. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ , \*\*\*\*:  $p < 0.0001$ . All data are presented as mean  $\pm$  SEM (n=8 mice per group).

333x247mm (300 x 300 DPI)

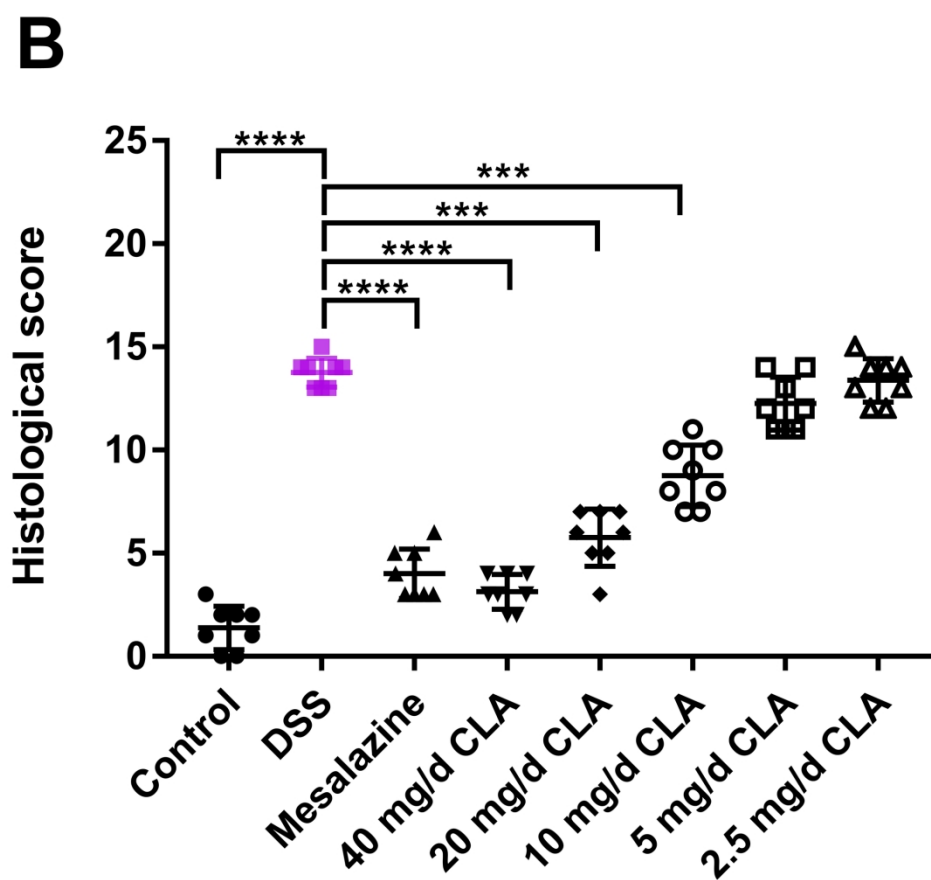


Fig.2B

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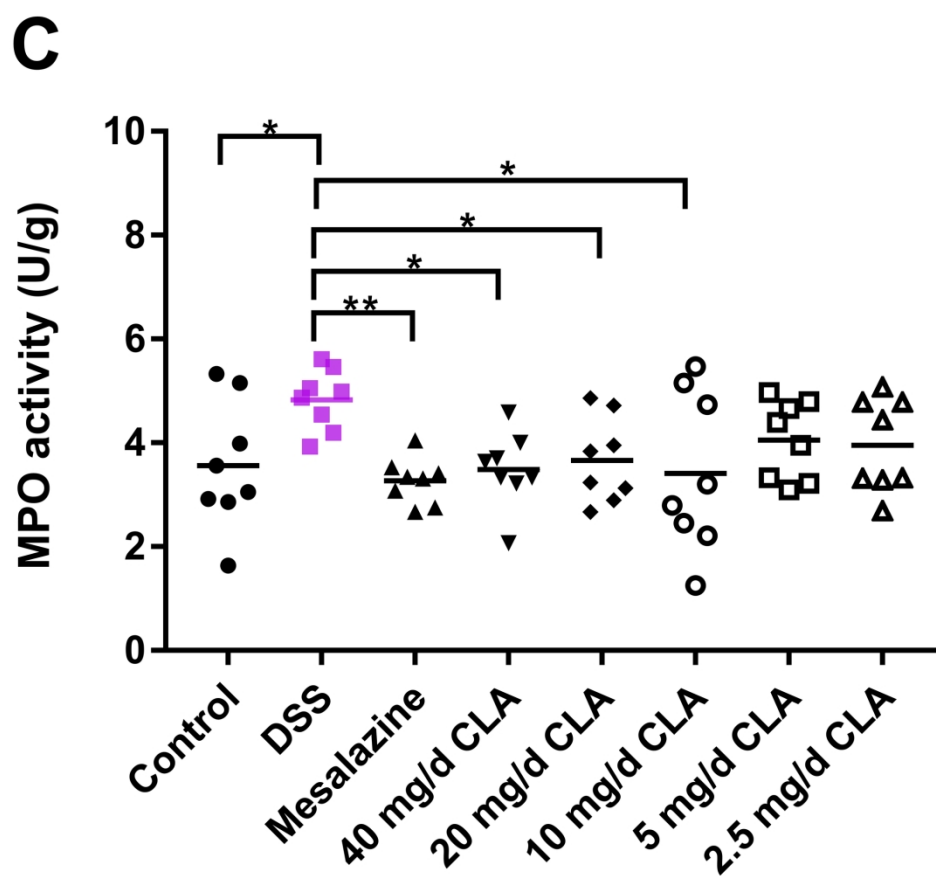


Fig.2C

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D

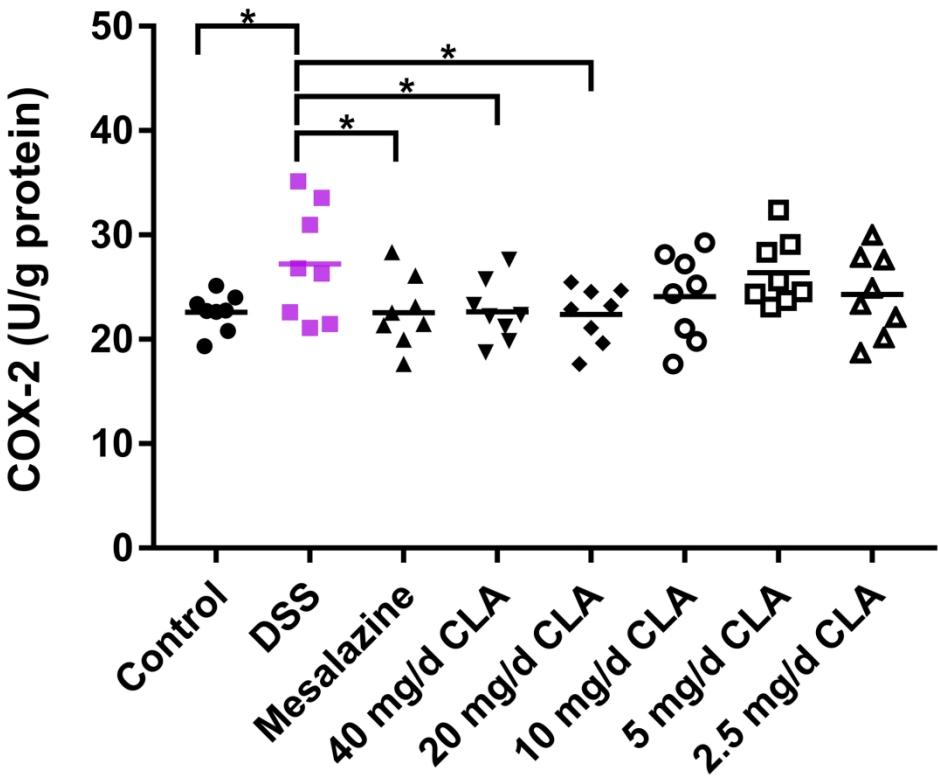


Fig.2D

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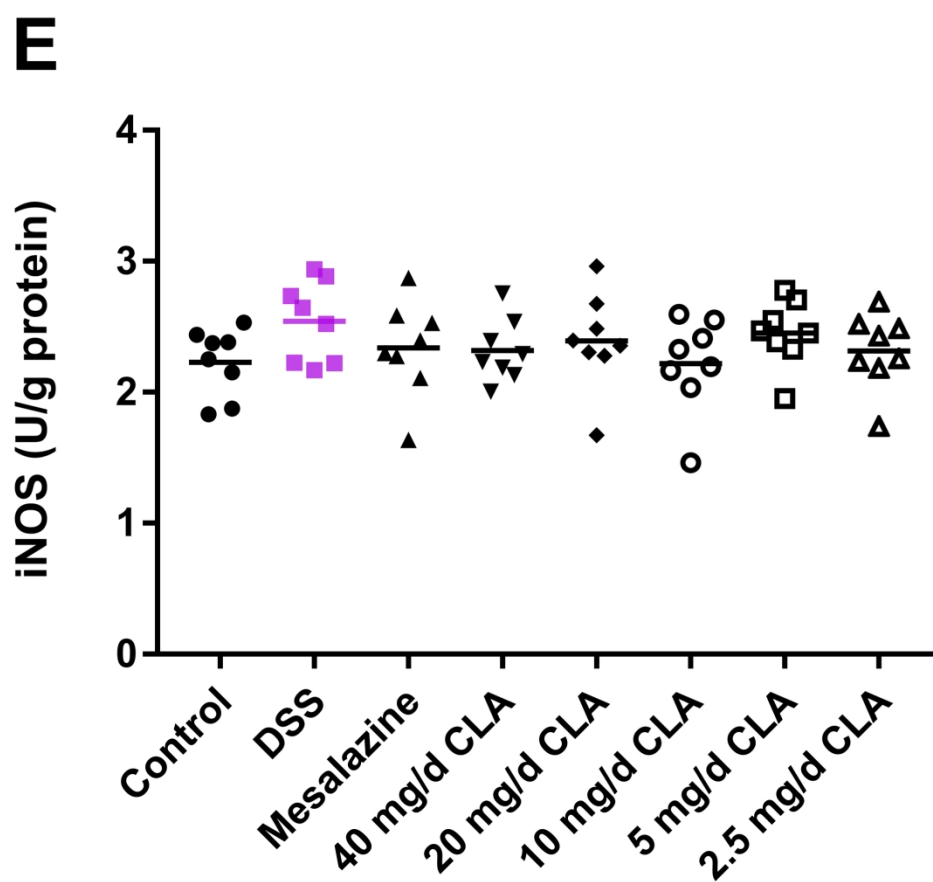


Fig.2E

194x186mm (300 x 300 DPI)



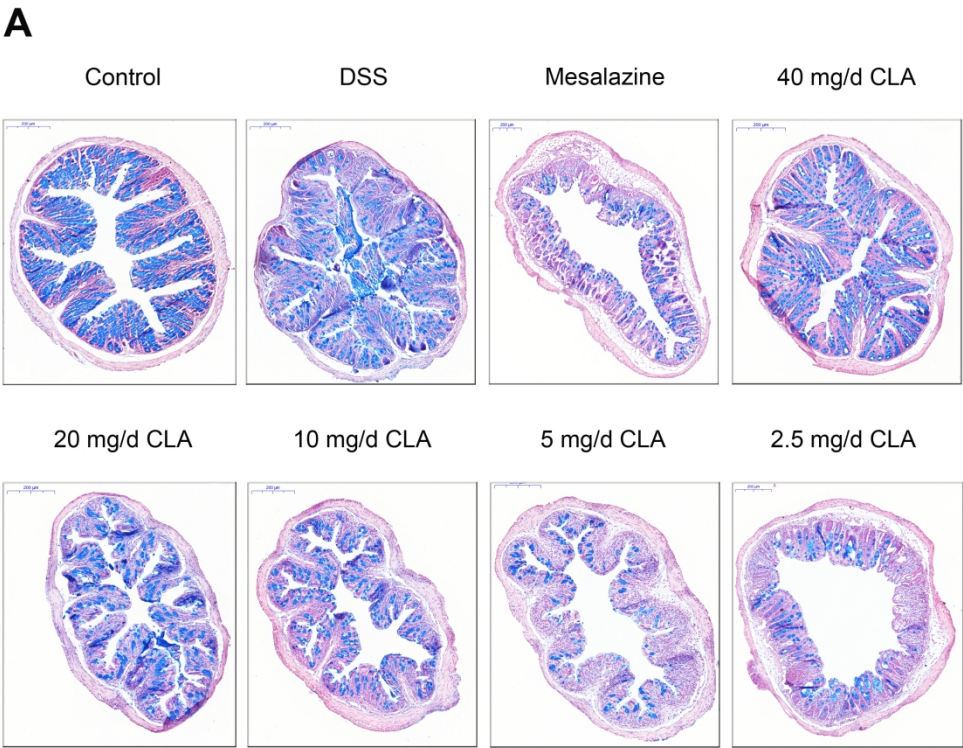


Fig.3 Effects of CLA on the mucous layer. (A) Alcian blue staining, Scale bar = 200  $\mu$ m (B) Histological sections of the colon (stained with PAS), Scale bars, 20  $\mu$ m, (C) Concentration of MUC2, (D) The number of goblet cells. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ . All data are presented as mean  $\pm$  SEM (n=8 mice per group).

267x204mm (300 x 300 DPI)

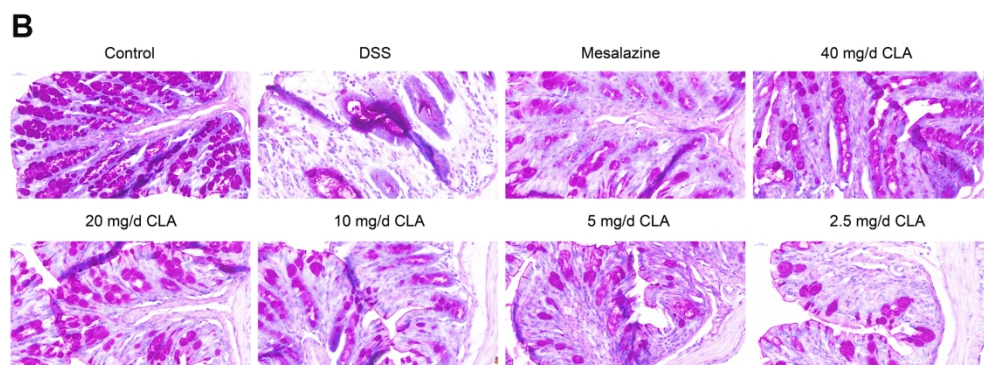


Fig.3B

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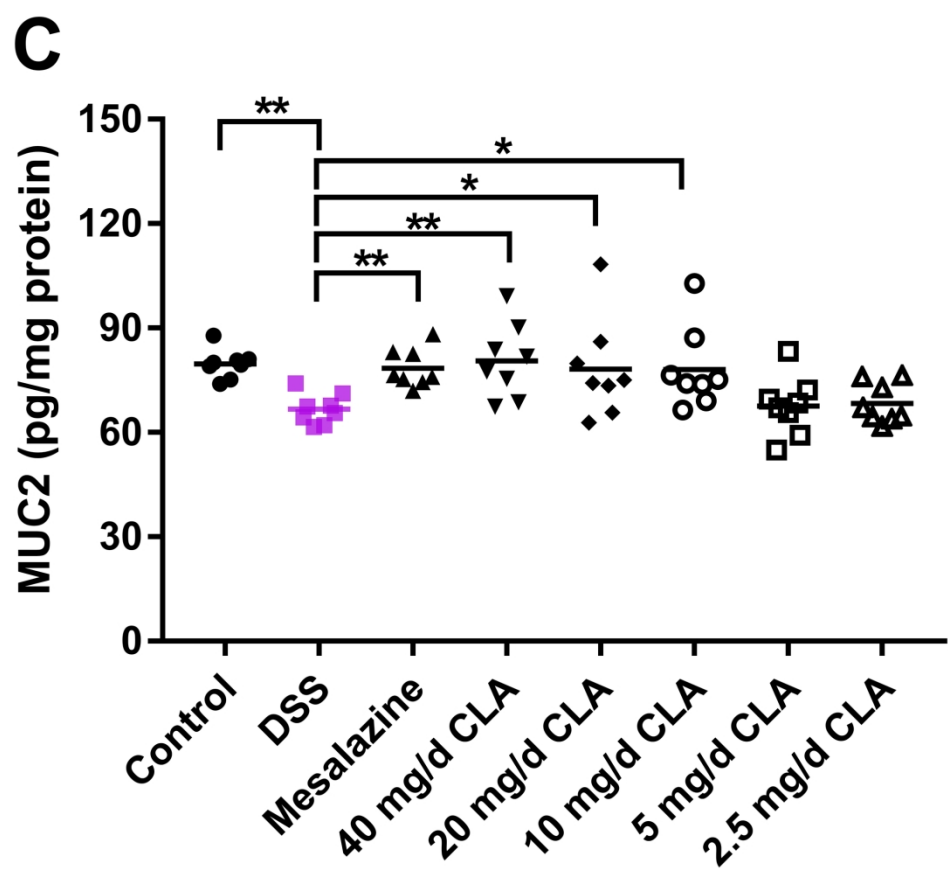


Fig.3C

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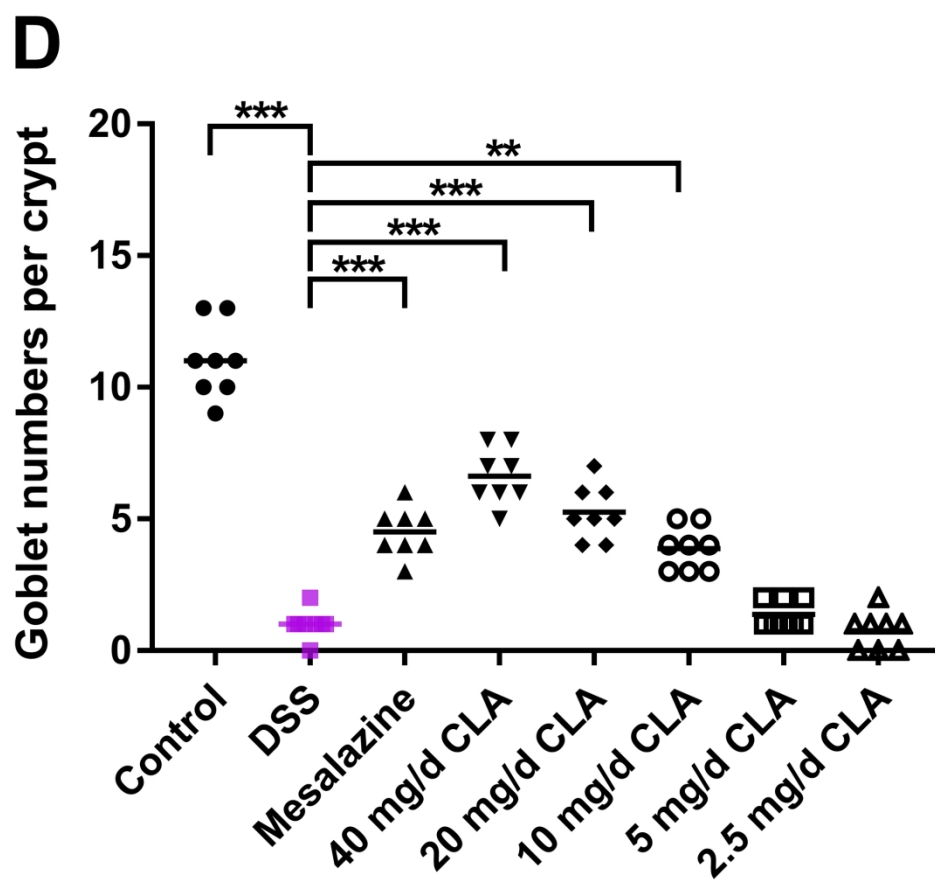


Fig.3D

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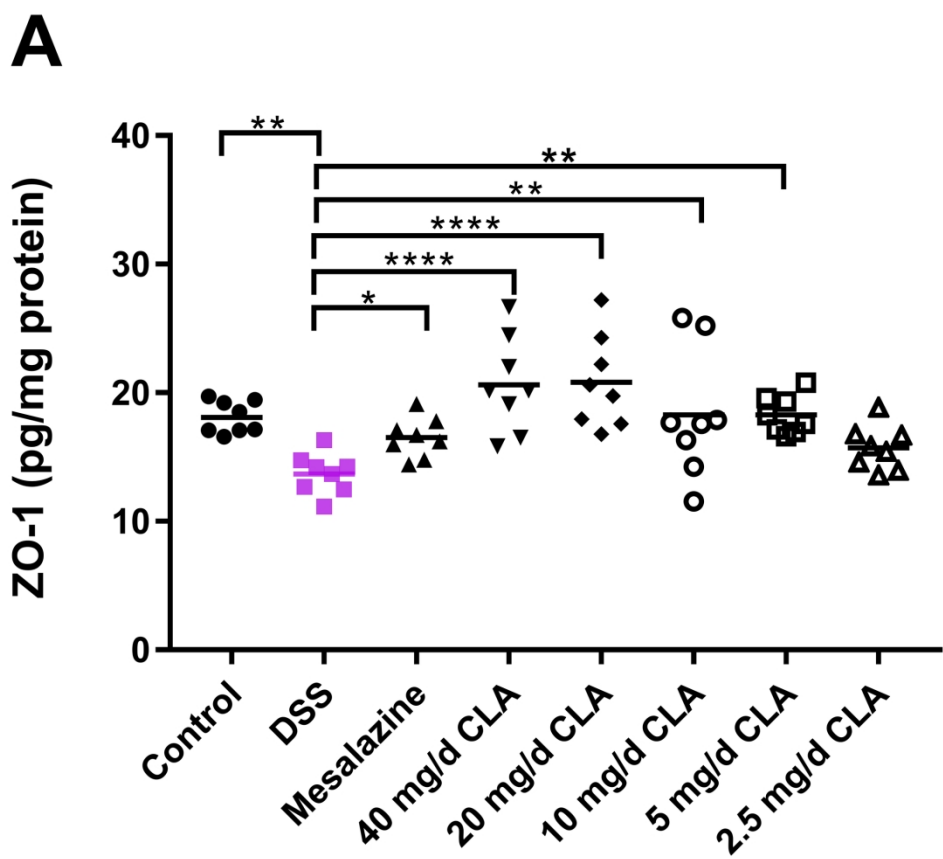


Fig.4 Effects of CLA on TJ proteins in colon and apoptosis of colonic epithelial cells. (A) ZO-1, (B) Occludin, (C) Claudin-3, (D) E-Cadherin1, (E) TEM of intestinal epithelial tissues, tight junction (TJ), adheres junction (AJ), desmosome (De), microvilli (Mv). Scale bar = 1  $\mu$ m, (F) apoptosis of colonic epithelial cells. Scale bar = 200  $\mu$ m. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ , \*\*\*\*:  $p < 0.0001$ . All data are presented as mean  $\pm$  SEM (n=8 mice per group).

199x186mm (300 x 300 DPI)

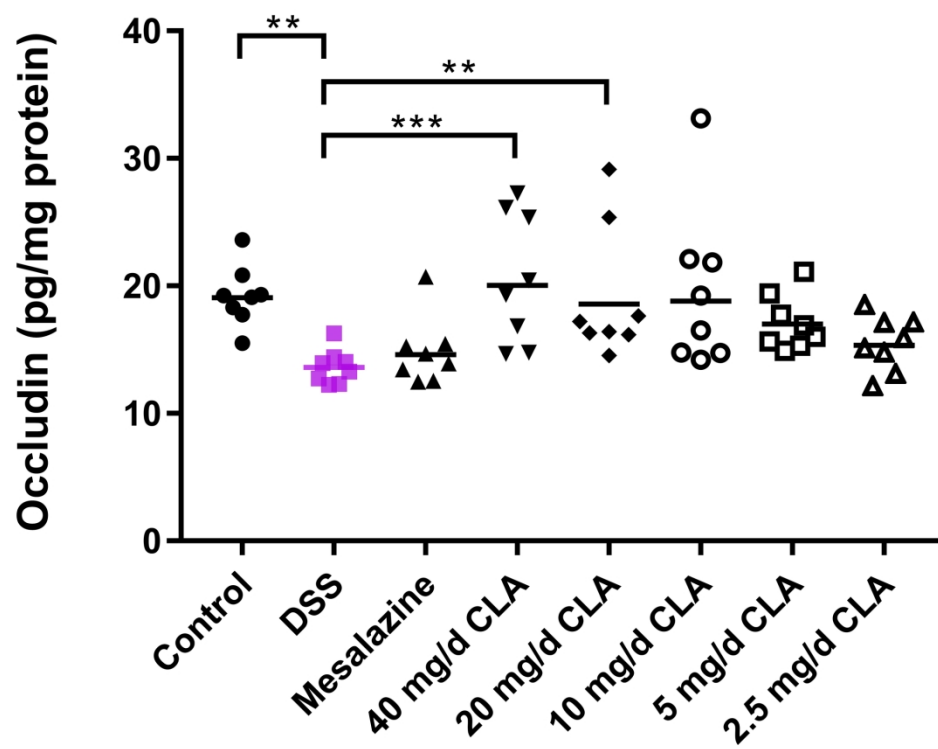
**B**

Fig.4B

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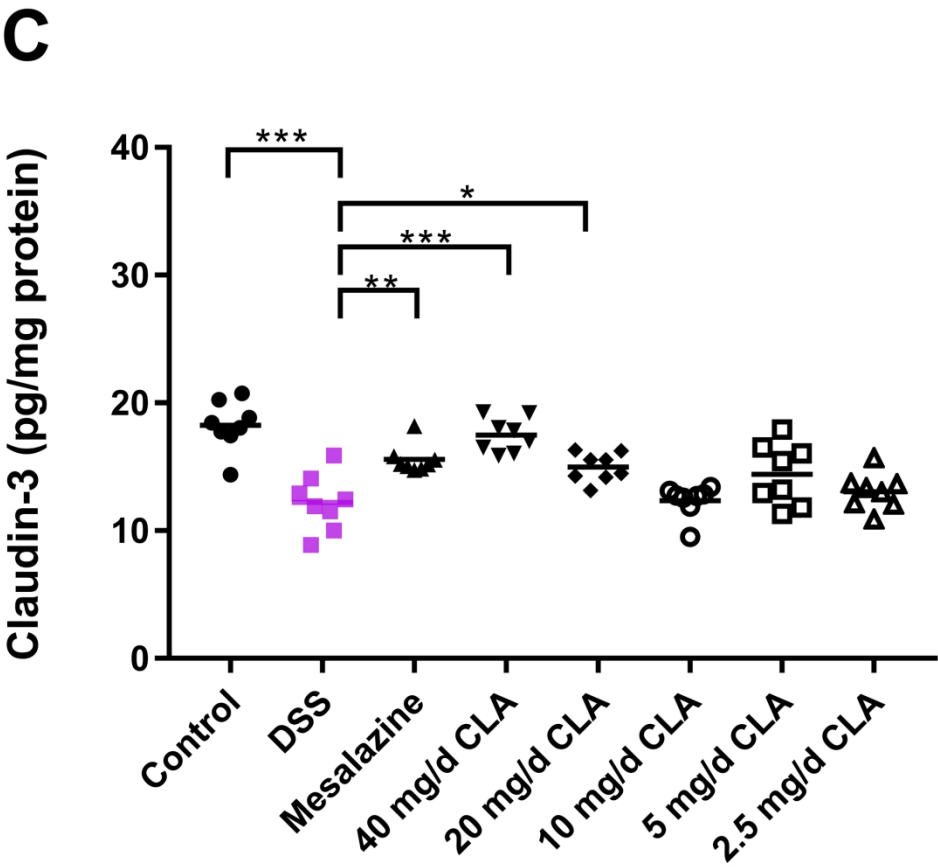


Fig.4C

199x188mm (300 x 300 DPI)

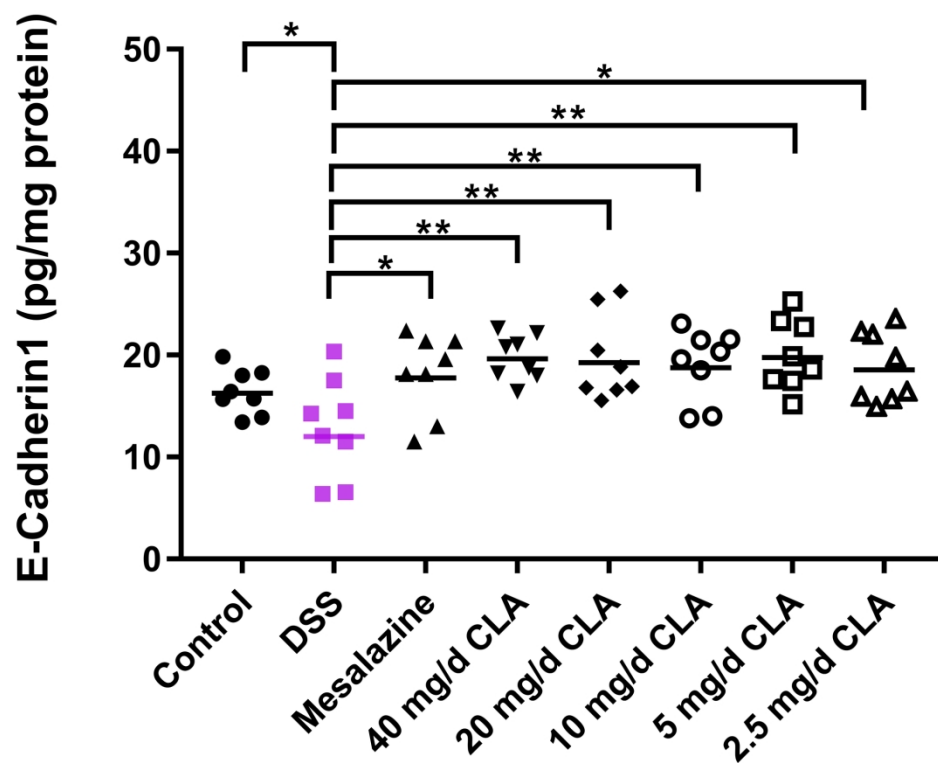
**D**

Fig.4D

199x192mm (300 x 300 DPI)



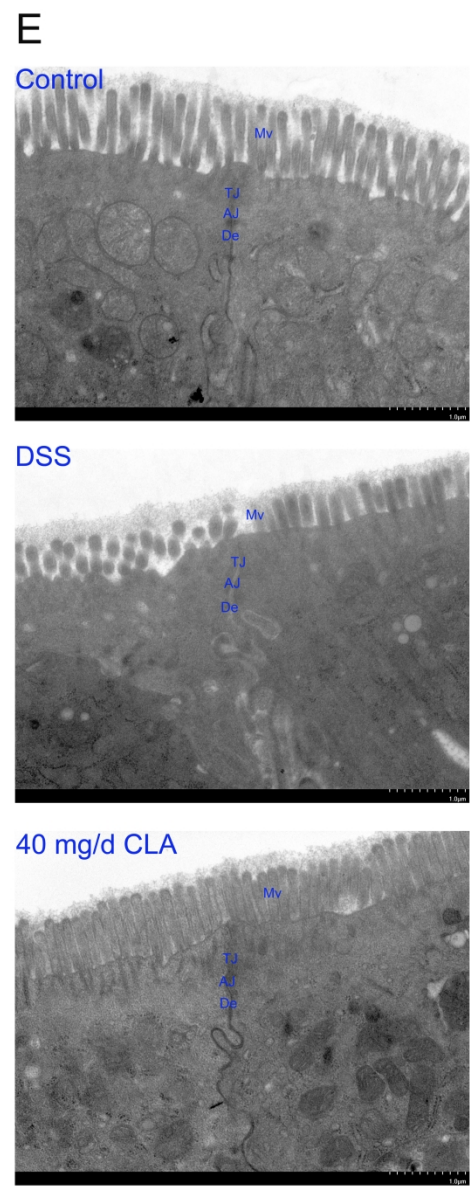


Fig.4E

F

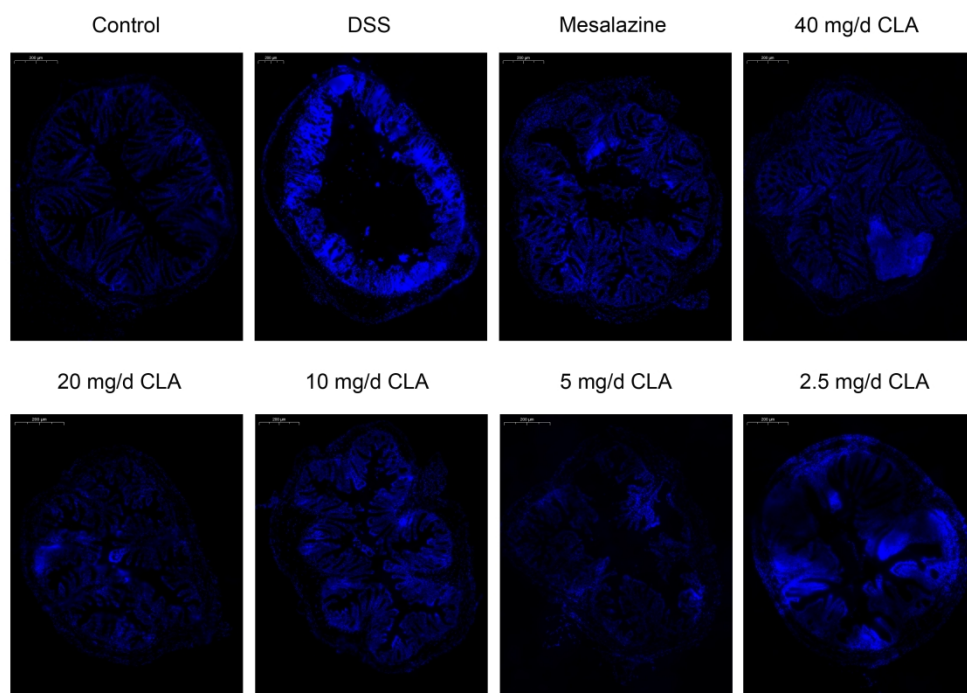


Fig.4F

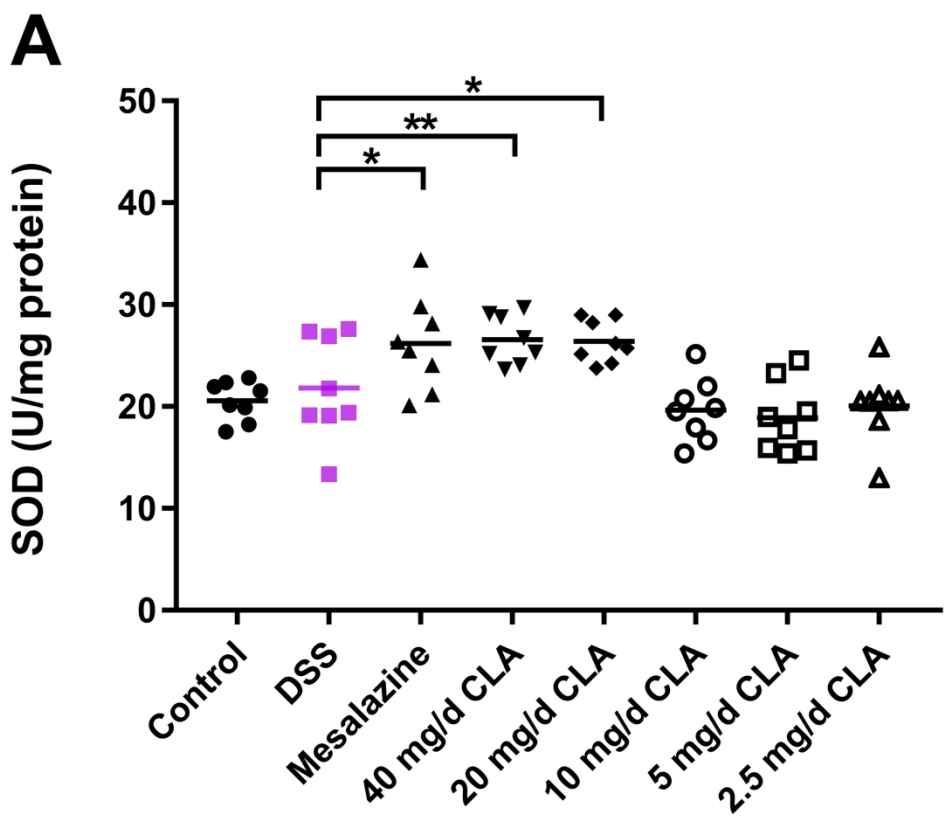


Fig.5. Effects of CLA on the activity of oxidative stress-related enzymes in colon. (A) SOD, (B) MDA, (C) GSH-PX, (D) CAT. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ . All data are presented as mean  $\pm$  SEM (n=8 mice per group).

199x178mm (300 x 300 DPI)

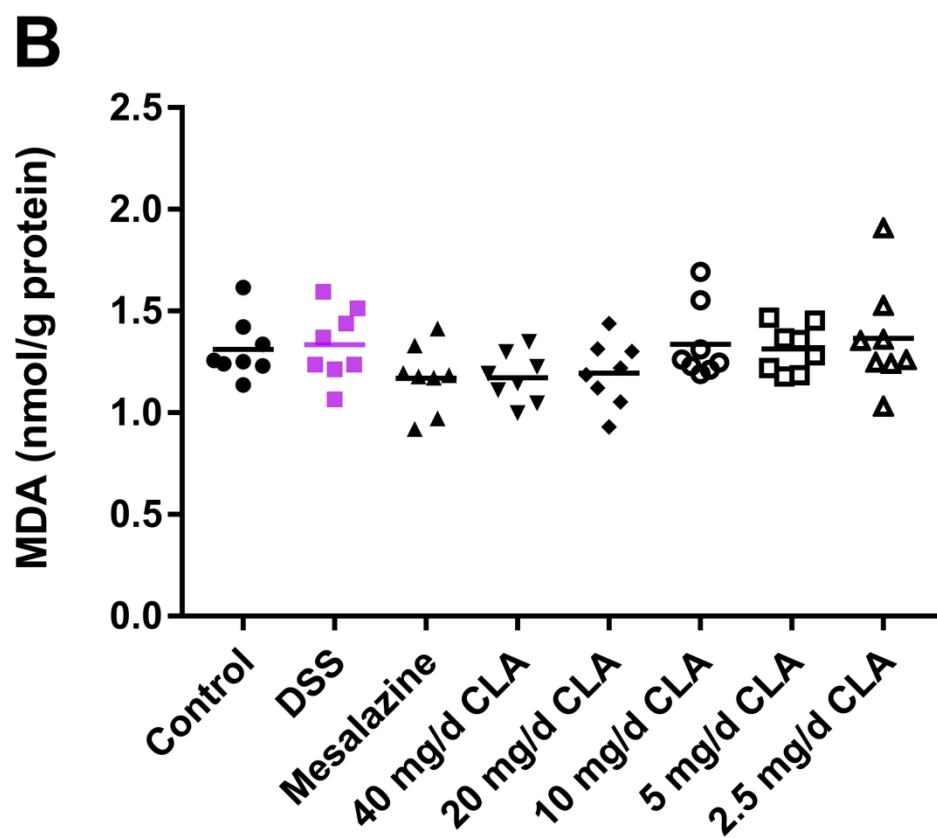


Fig.5B

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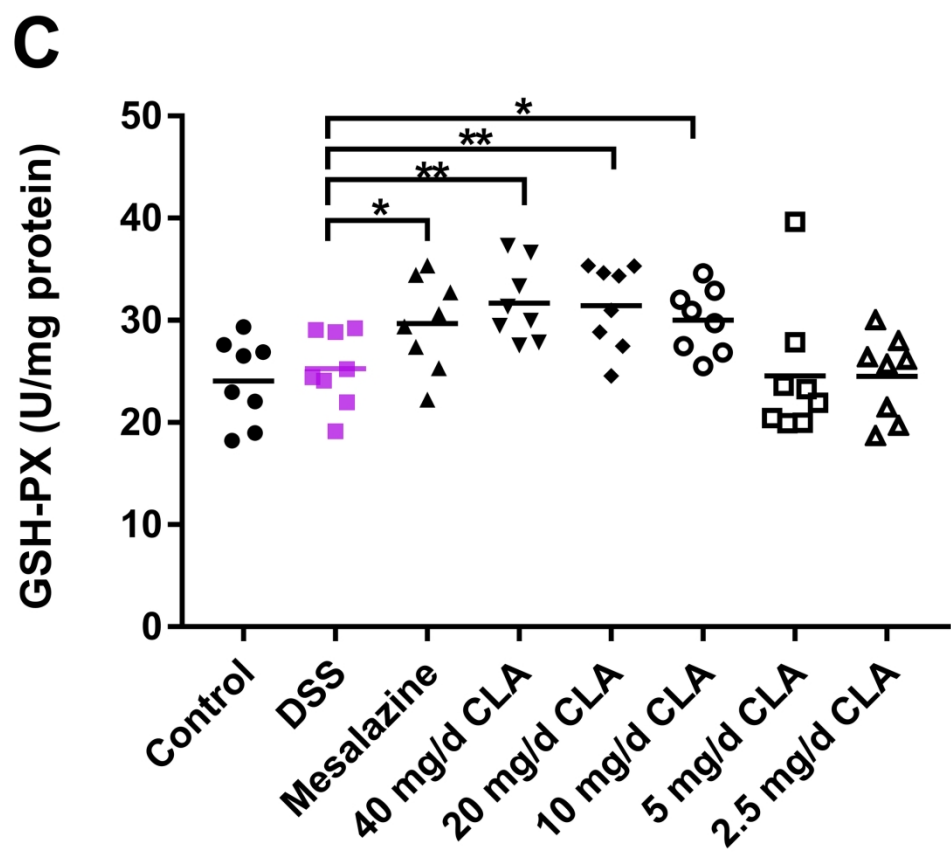


Fig.5C

199x185mm (300 x 300 DPI)

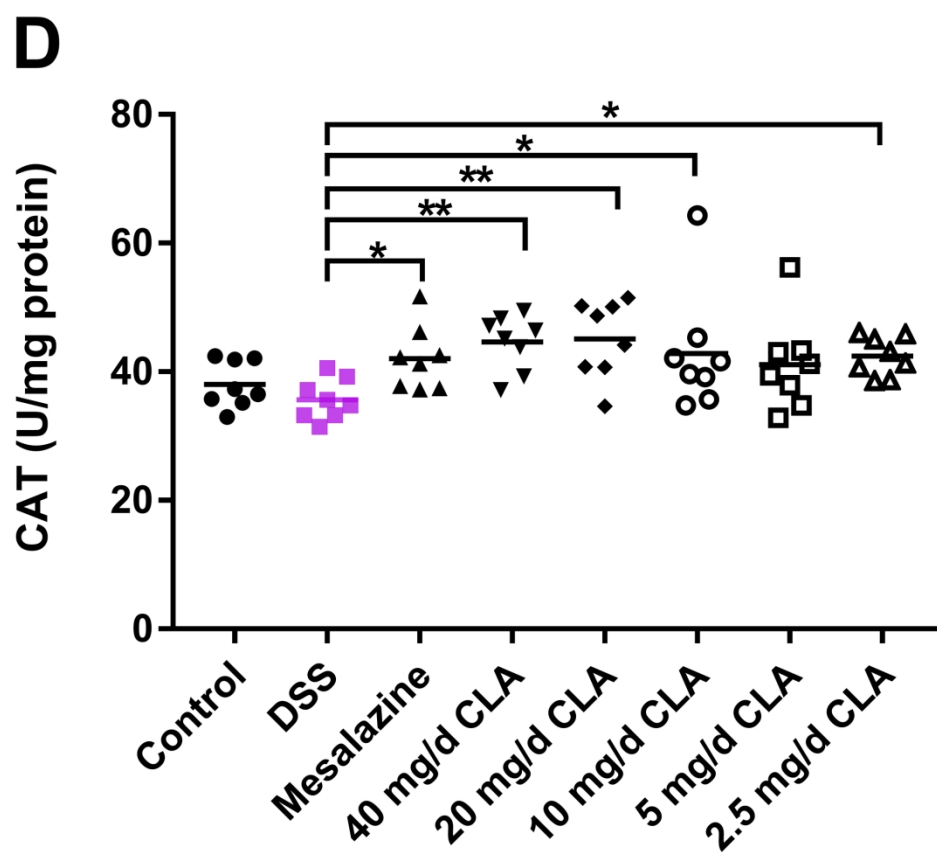


Fig.5D

199x185mm (300 x 300 DPI)

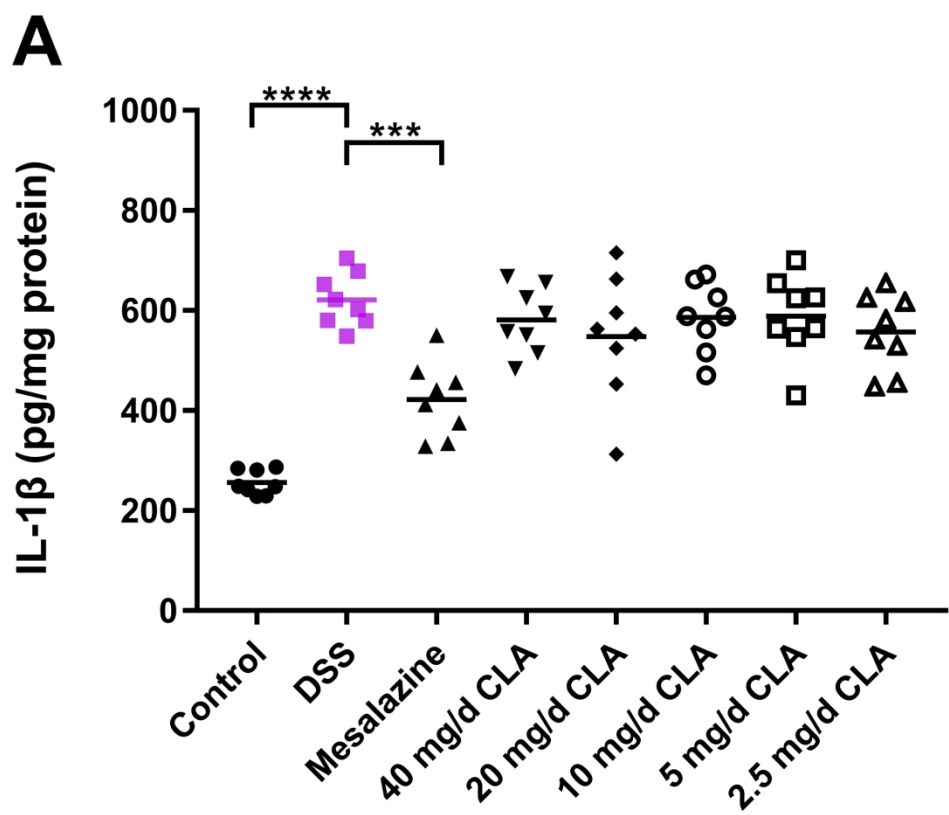


Fig.6. Effects of CLA on inflammatory cytokines in colonic tissue. (A) IL-1 $\beta$ , (B) TNF- $\alpha$ , (C) IL-6, (D) IL-10 and (E) PPAR $\gamma$ . \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ , \*\*\*\*:  $p < 0.0001$ . All data are presented as mean  $\pm$  SEM (n=8 mice per group).

203x180mm (300 x 300 DPI)

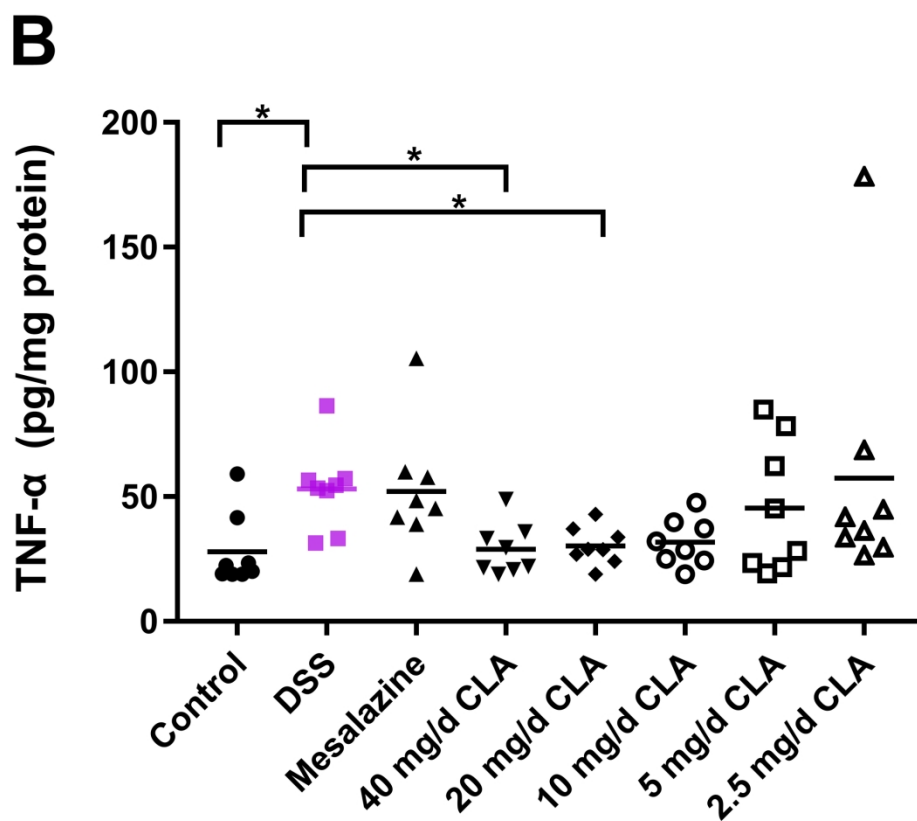


Fig.6B

203x182mm (300 x 300 DPI)



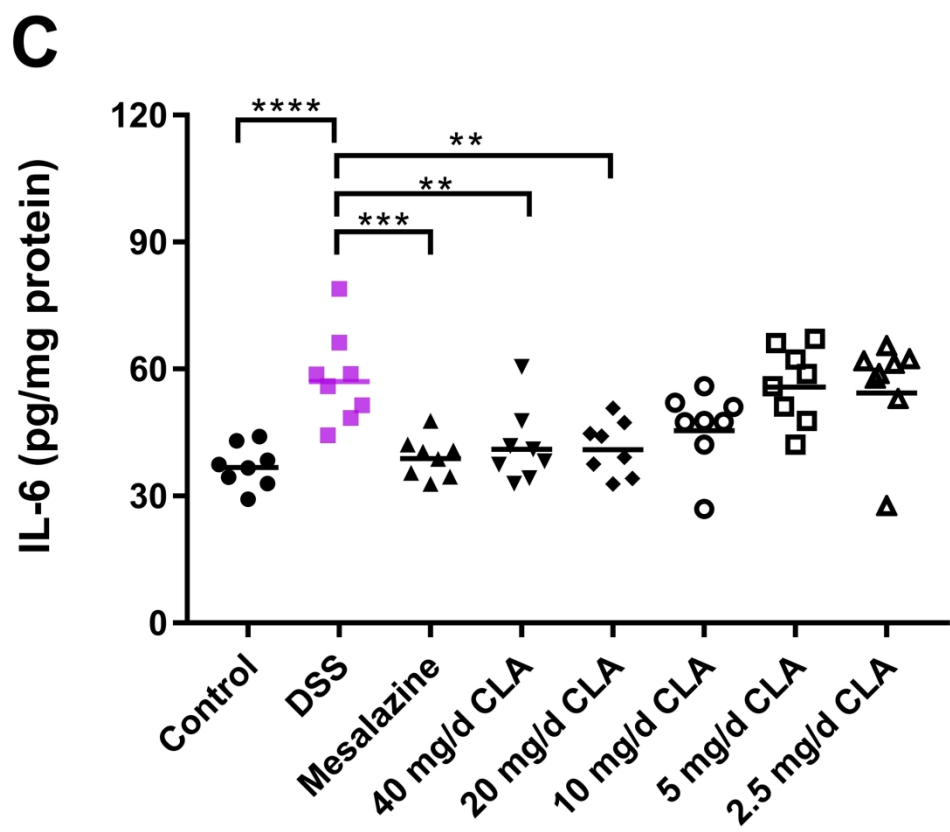


Fig.6C

203x183mm (300 x 300 DPI)

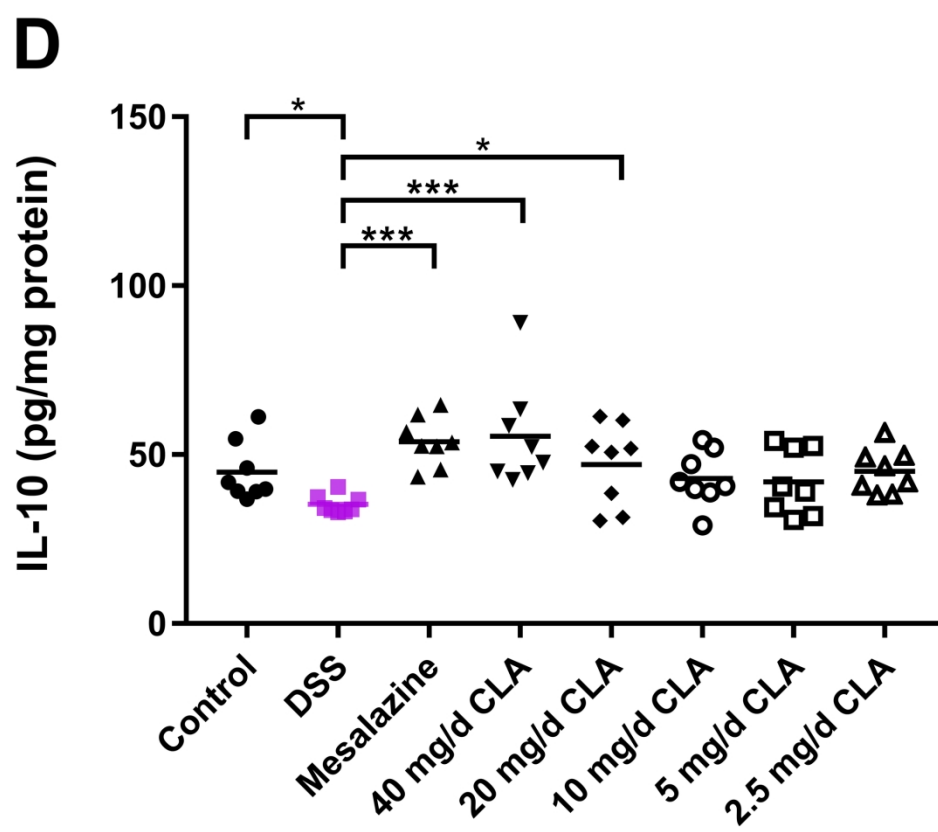


Fig.6D

203x183mm (300 x 300 DPI)

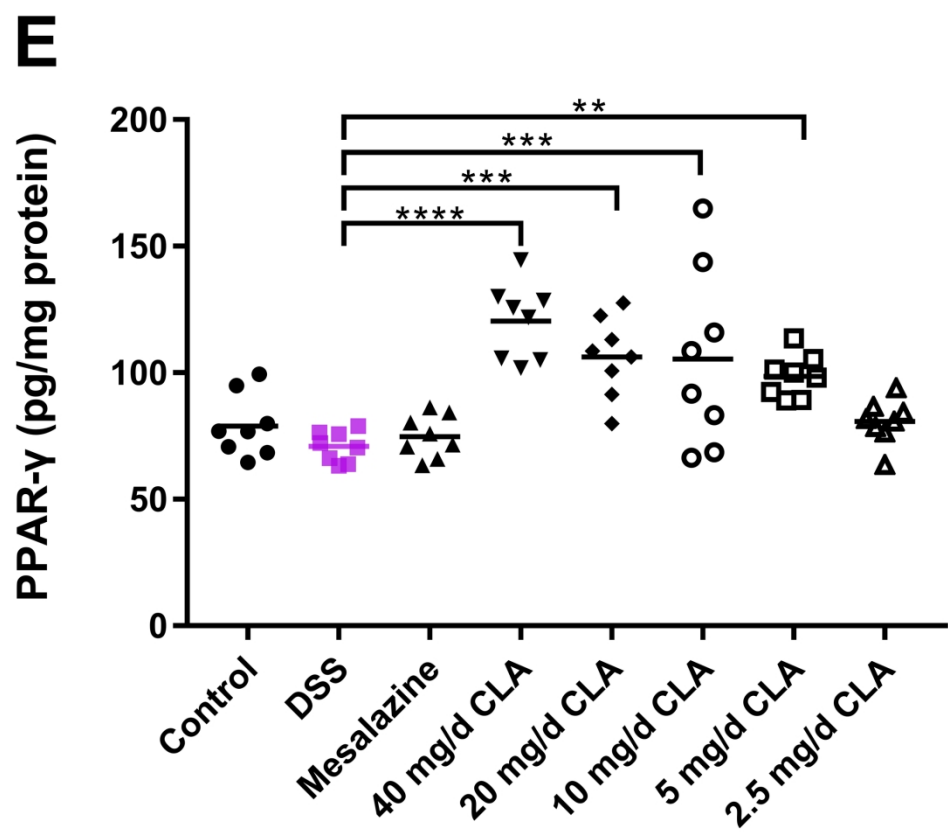


Fig.6E

203x184mm (300 x 300 DPI)

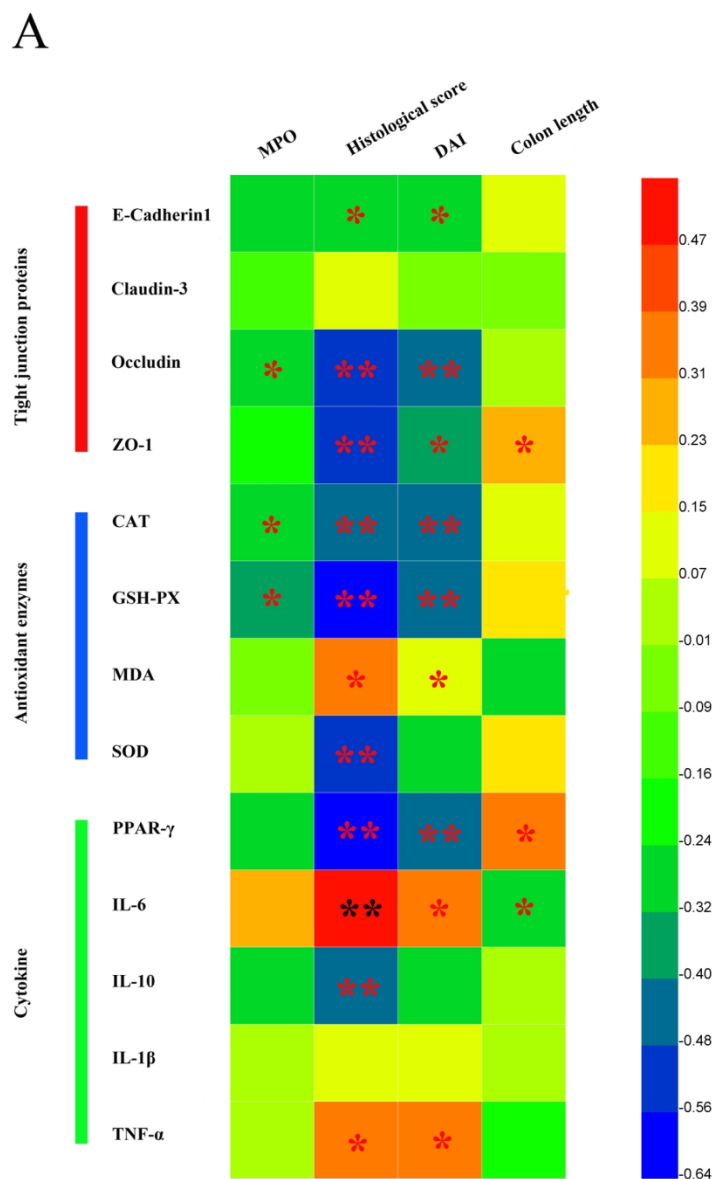


Fig.7. CLA concentration in different tissues and correlation of CLA and colitis indices. (A) Correlation analysis of colitis indices and different parameters. (B-D) CLA concentration in the colonic, blood and liver.

(E-H) The interdependent quantitative relationships between the colonic CLA concentration and inflammatory markers, DAI, histological scores, colon length and MPO. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ , \*\*\*\*:  $p < 0.0001$ . All data are presented as mean  $\pm$  SEM (n=8 mice per group).

105x164mm (300 x 300 DPI)

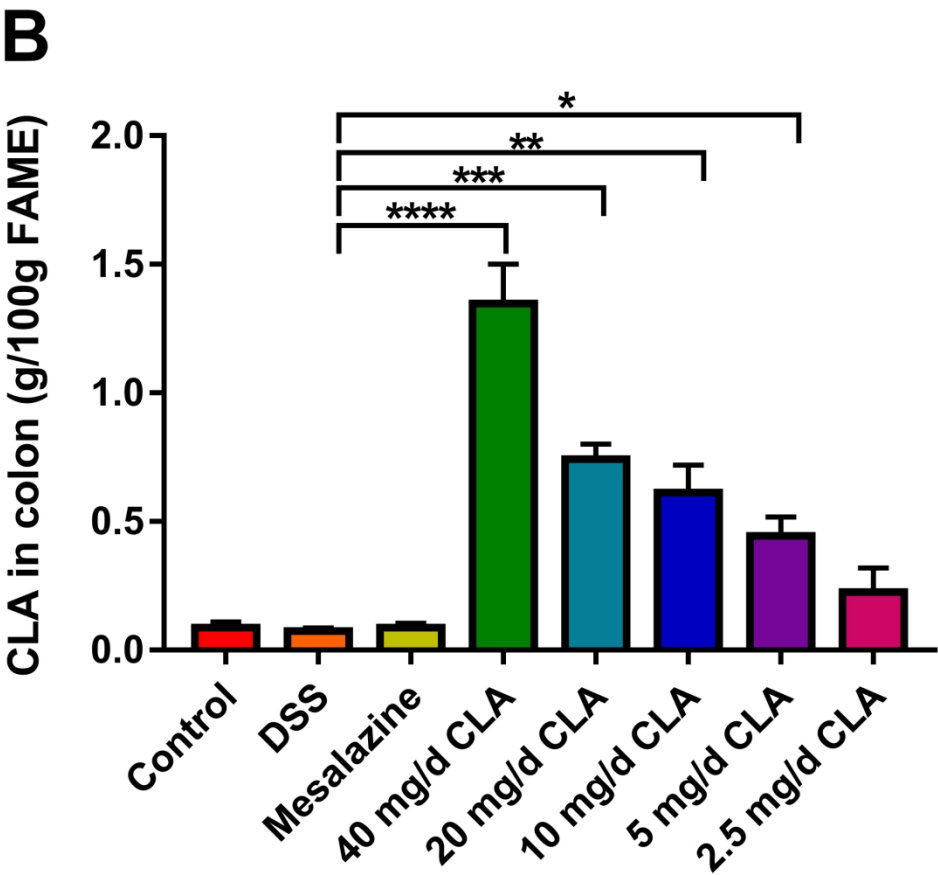


Fig.7B

197x189mm (300 x 300 DPI)

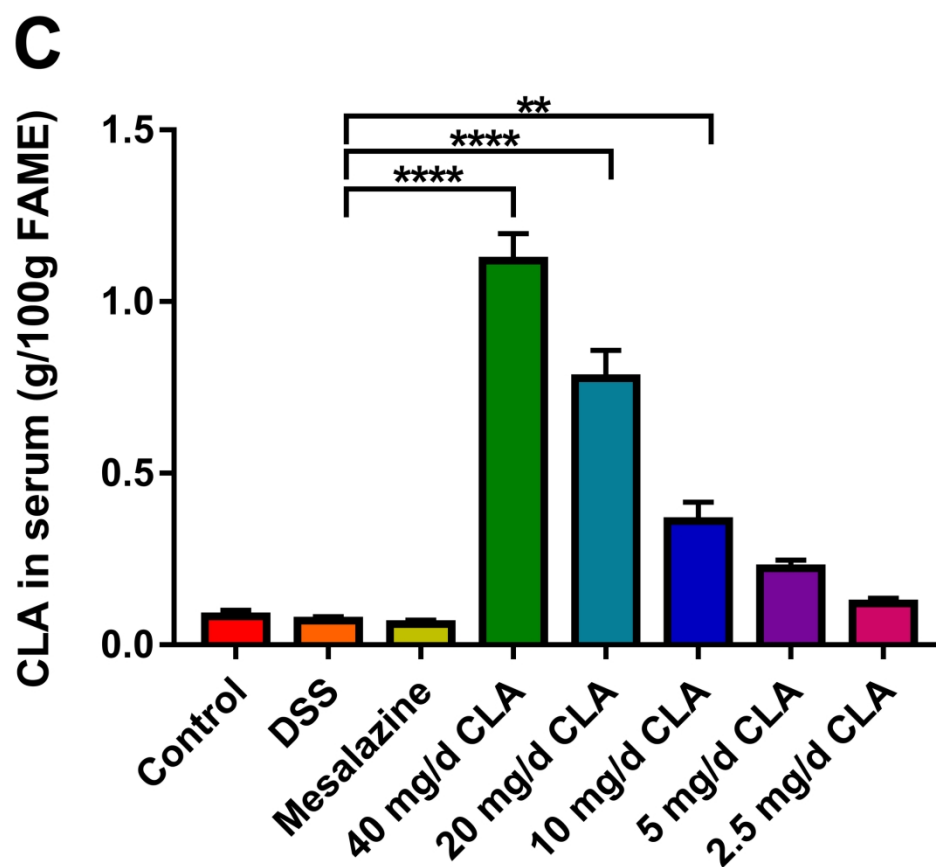


Fig.7C

197x186mm (300 x 300 DPI)

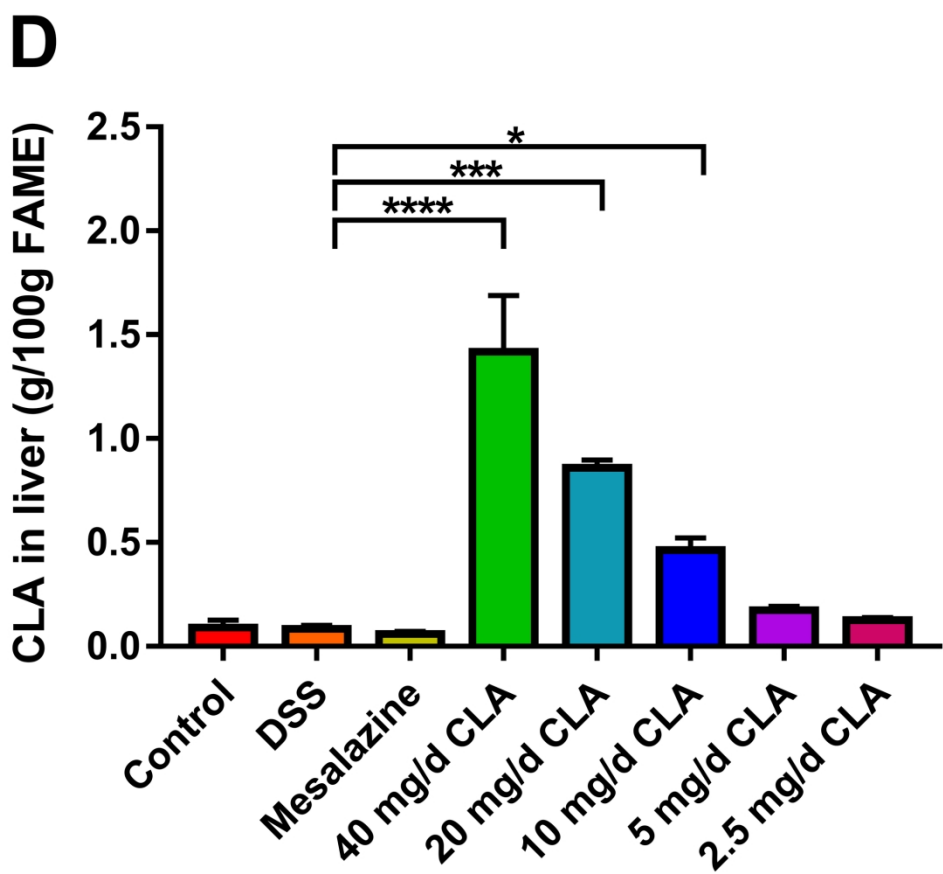


Fig.7D

197x188mm (300 x 300 DPI)

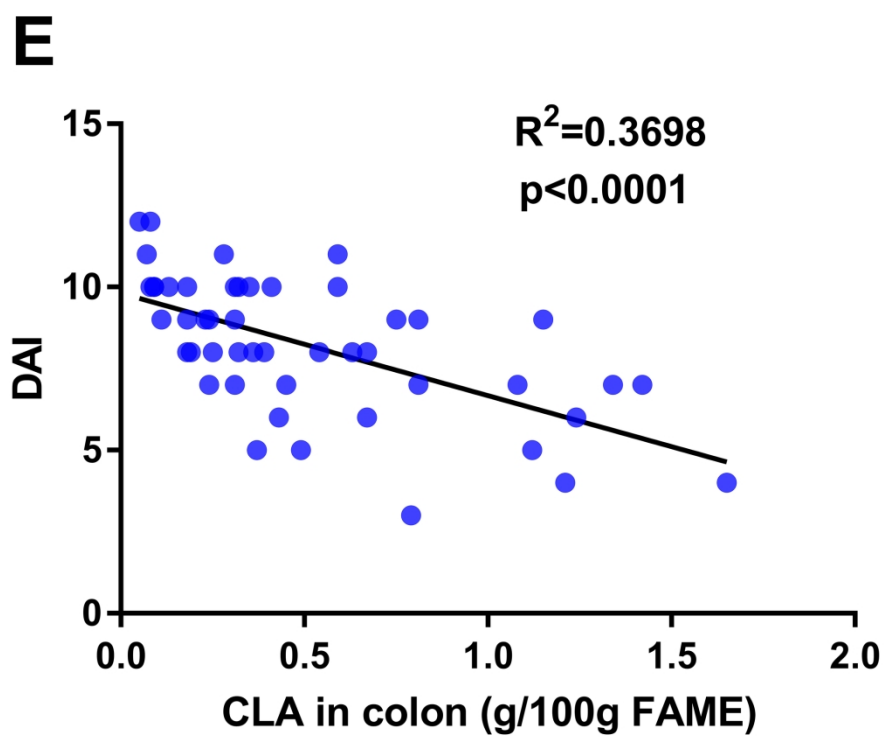


Fig.7E

207x166mm (300 x 300 DPI)



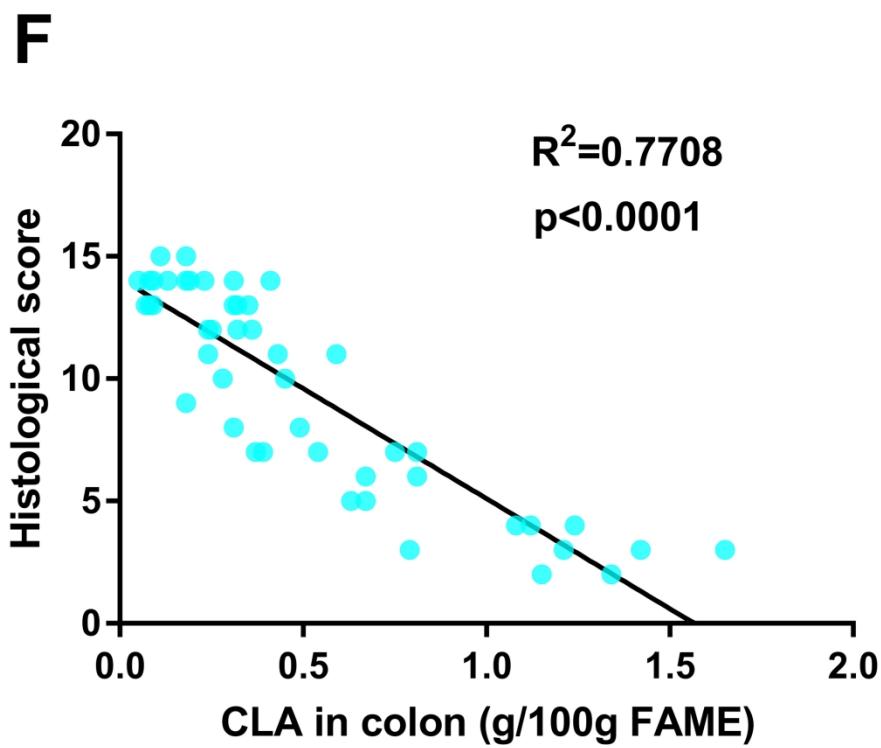


Fig.7F

207x169mm (300 x 300 DPI)

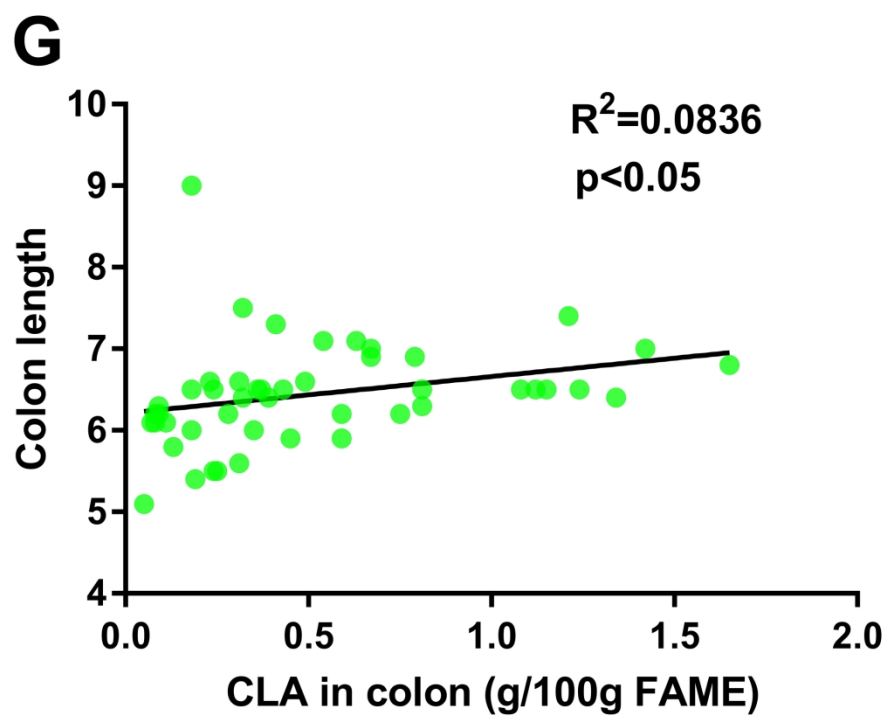


Fig.7G

207x162mm (300 x 300 DPI)

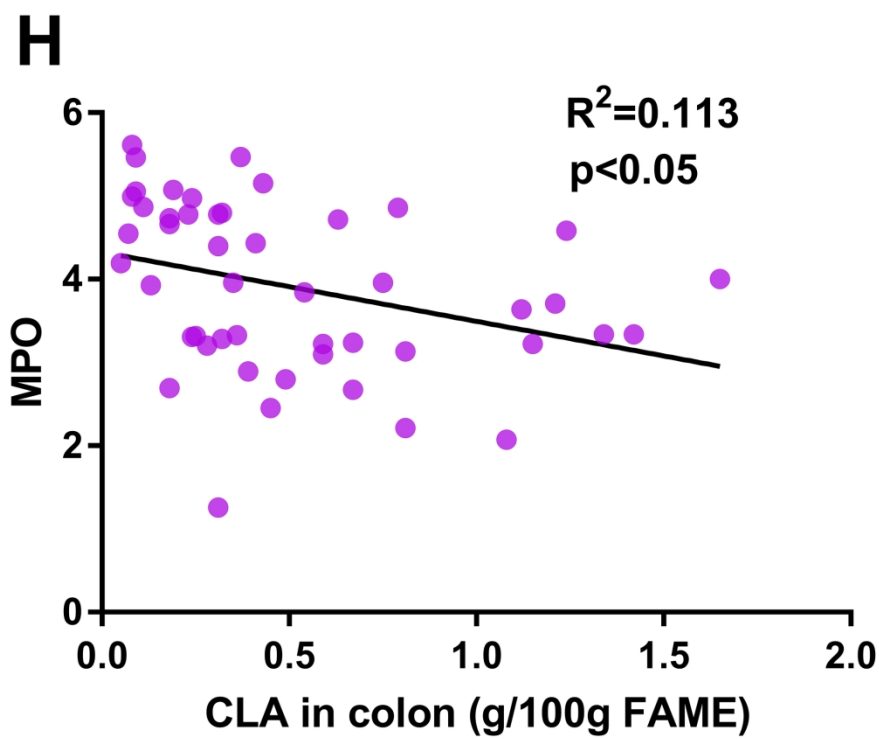


Fig.7H

207x167mm (300 x 300 DPI)

A

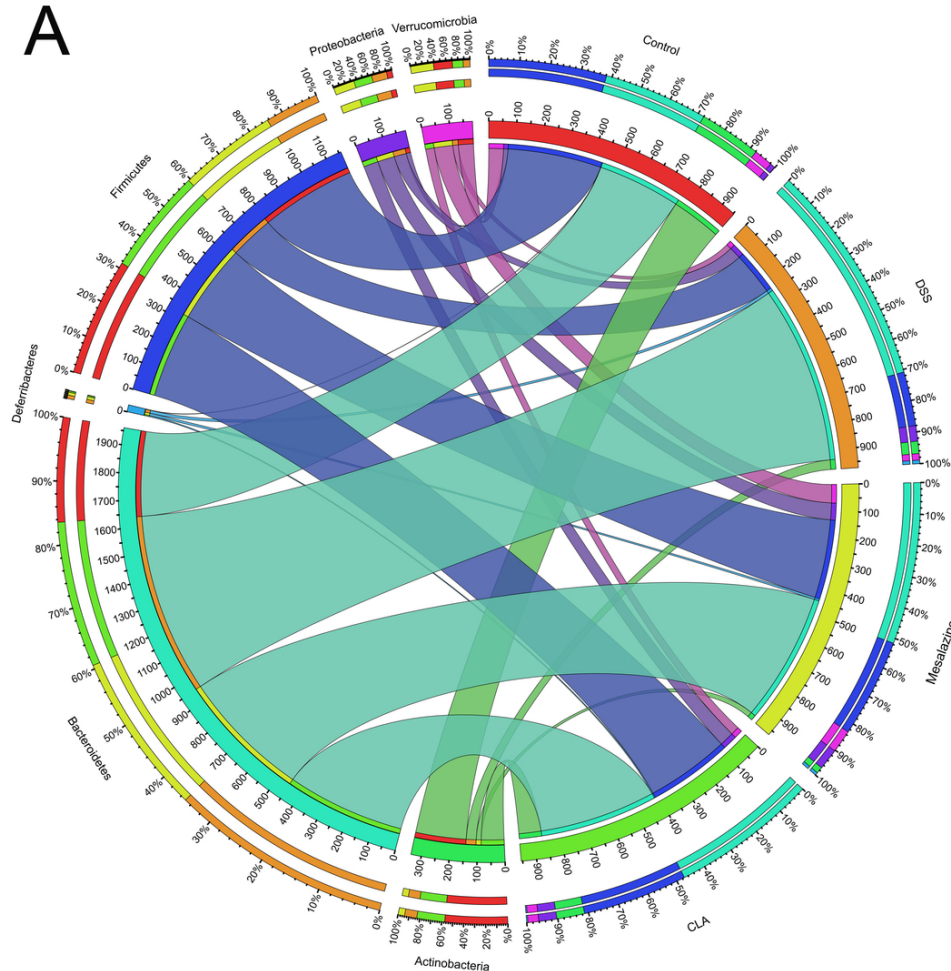


Fig.8 Evaluation of illumina MiSeq sequencing data showing that CLA could modulate the overall structure of gut microbiota. (A) Microbial distribution at the phylum level, (B) Alpha diversity indicated by Chao1 index, (C) Shannon index, (D) PCoA, with extended functionality for labeling groups, with normal probability ellipsoids for different groups, (E) Network diagram of sample and OTU. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ , \*\*\*\*:  $p < 0.0001$ . All data are presented as mean  $\pm$  SEM (n=8 mice per group).

93x92mm (300 x 300 DPI)

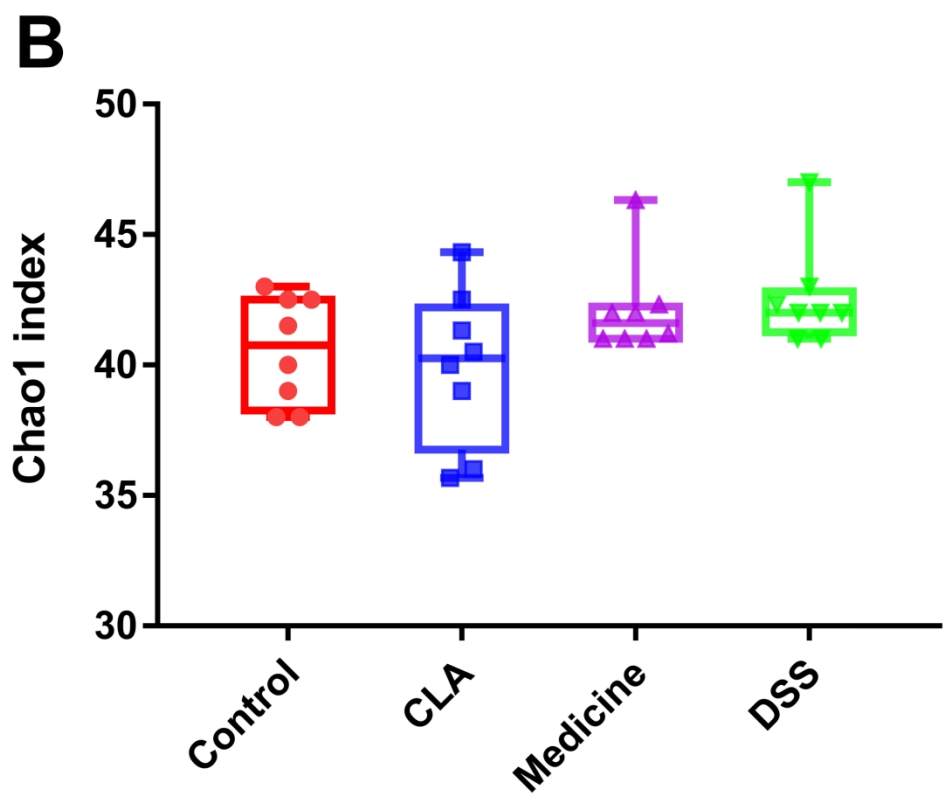


Fig.8B

194x170mm (300 x 300 DPI)

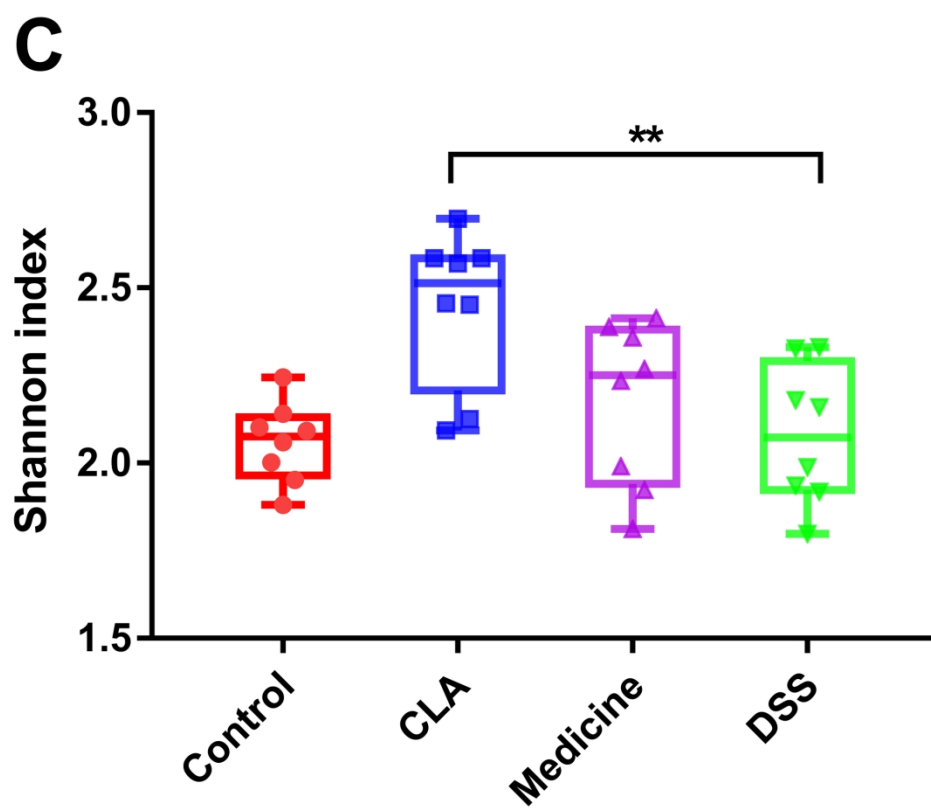


Fig.8C

194x172mm (300 x 300 DPI)

D

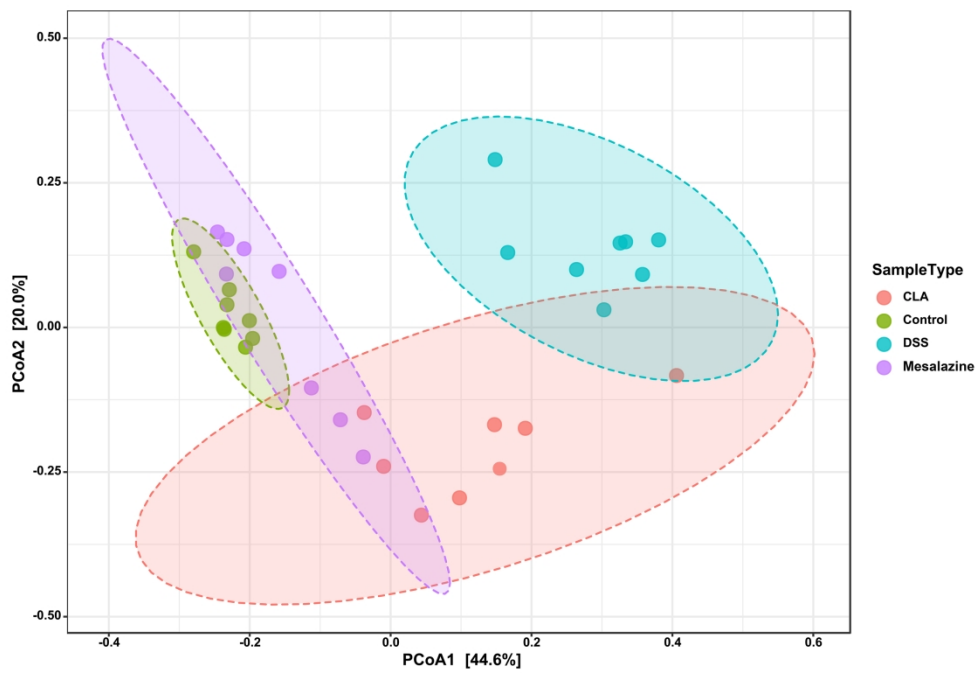


Fig.8D

250x202mm (300 x 300 DPI)

E

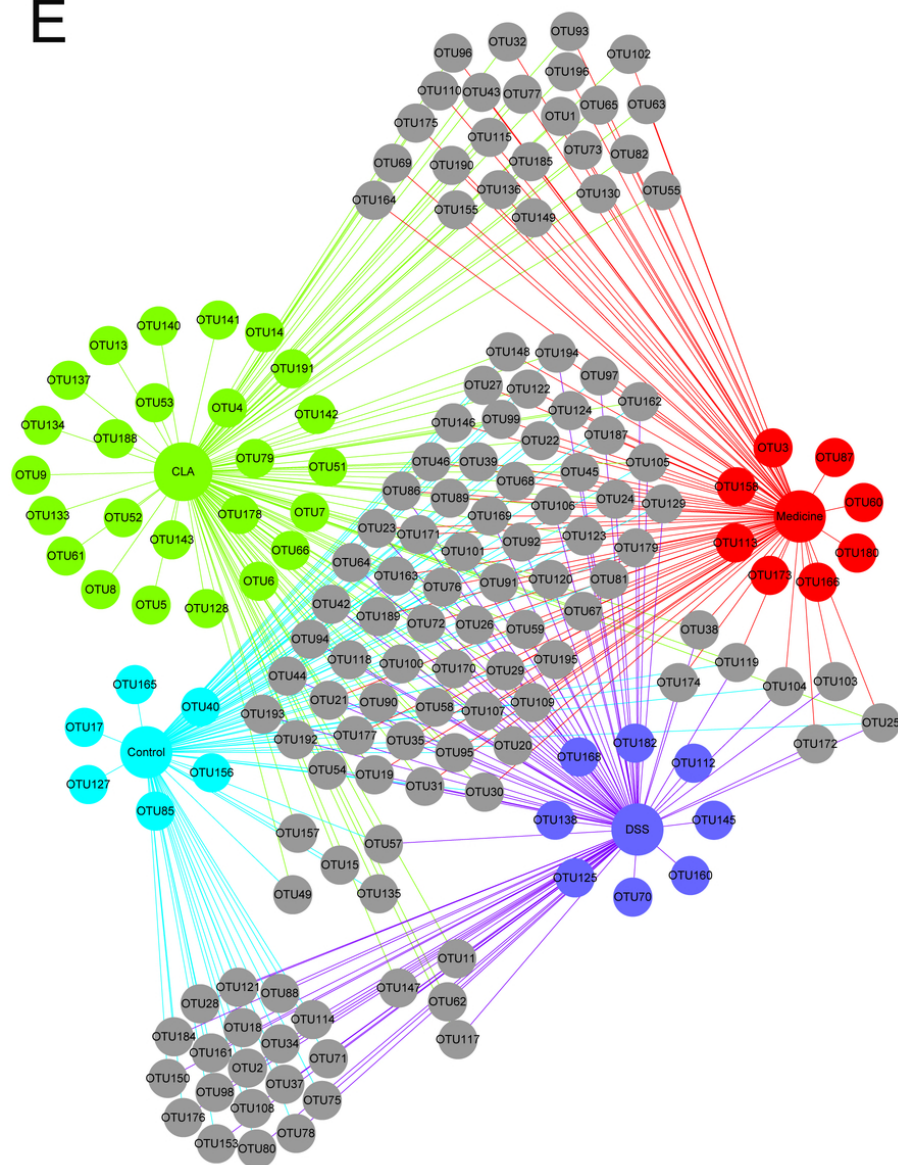


Fig.8E

75x99mm (300 x 300 DPI)



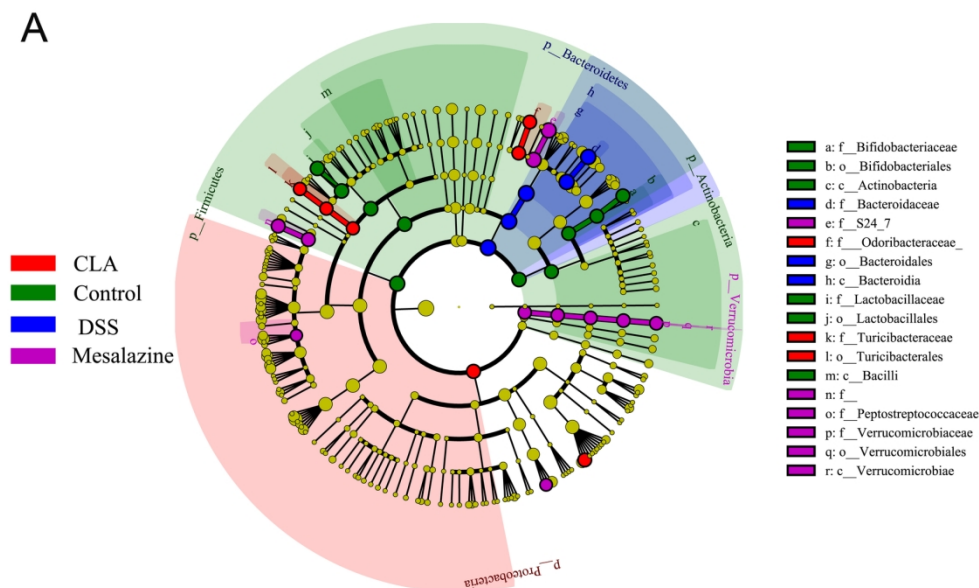


Fig.9 Effects of CLA on dominant microorganisms. (A) Cladogram. (B) Distribution histogram based on LDA, with a log LDA score above 3.0. Significant taxa are labeled and annotated with tags in the right panel. (C) Relative abundance of S24-7, Bifidobacterium, Lactobacillus, Akkermansia, Bacteroides and Odoribacter. (D) Effect of CLA treatment on bacterial-interaction patterns of the validation cohort. Bacterial abundances were analyzed using Spearman’s test. Only significant correlations ( $p$ -value  $< 0.05$ ,  $|R2| > 0.6$ ) are displayed with an edge. The edge colors indicate positive (red) or negative (green) correlations, which depended on Spearman’s correlation coefficient. The node size represents the weight. (E) Correlation analysis of the concentration of colonic CLA, significant taxa, colitis indices, tight junction proteins, antioxidant enzymes and cytokine in colon. Only significant correlations ( $p$ -value  $< 0.05$ ,  $|r2| > 0.6$ ) are displayed with an edge. The edge colors indicate positive (red) or negative (green) correlations, which depended on Spearman’s correlation coefficient. The node size represents the weight.  $n=8$  mice per group.

157x94mm (300 x 300 DPI)

B

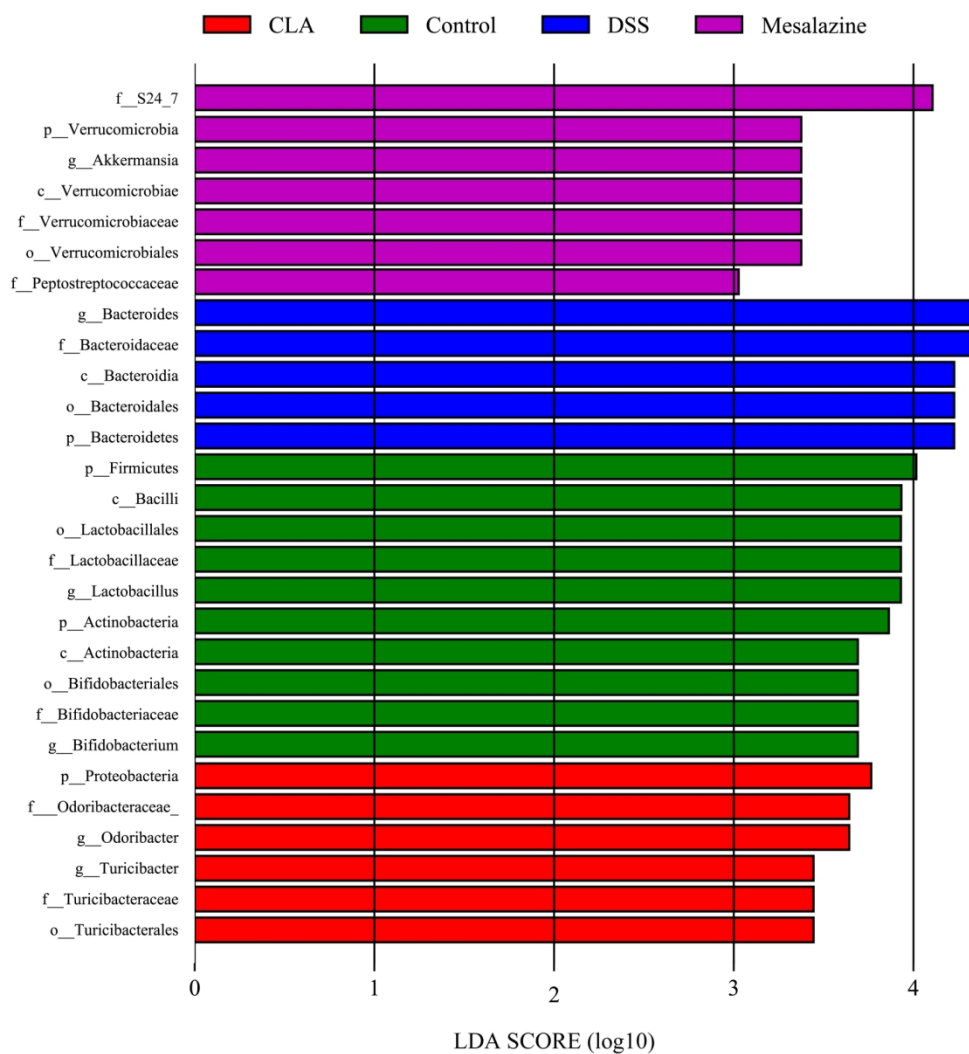


Fig.9B

152x172mm (300 x 300 DPI)

C

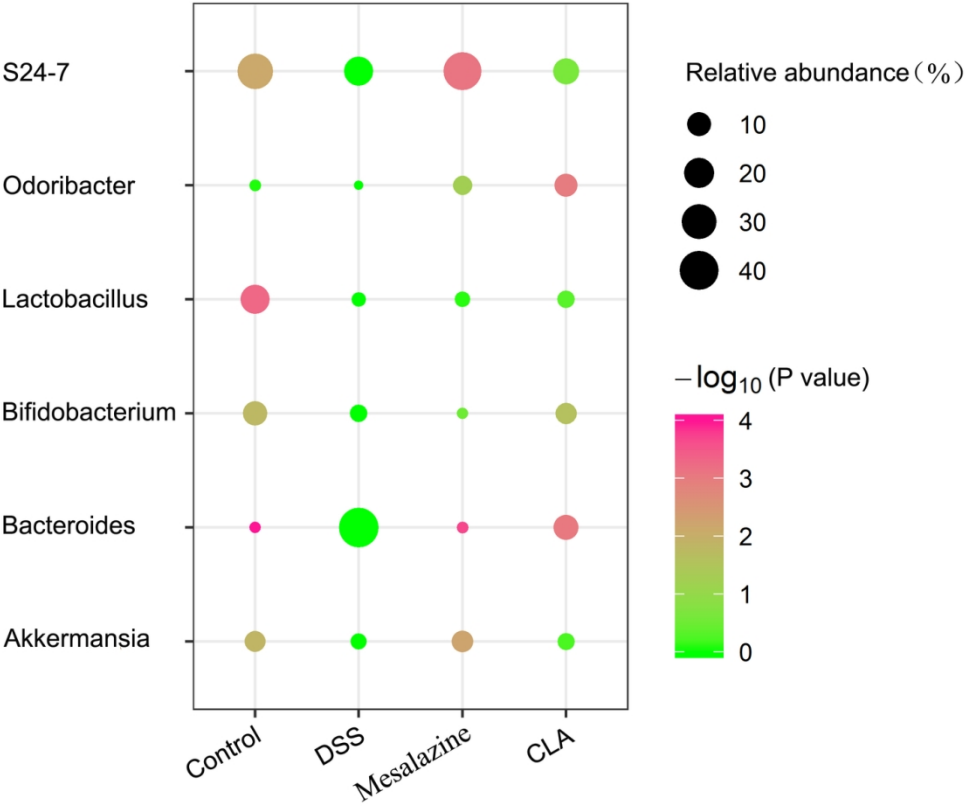


Fig.9C

125x113mm (300 x 300 DPI)

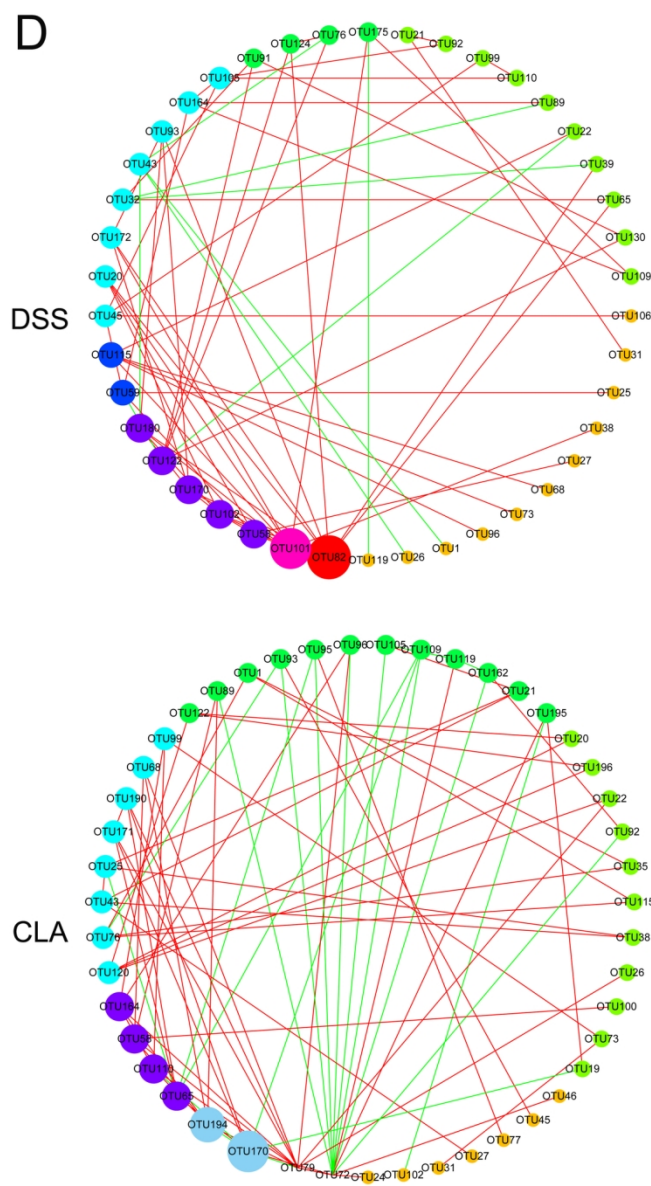


Fig.9D

98x176mm (300 x 300 DPI)

E

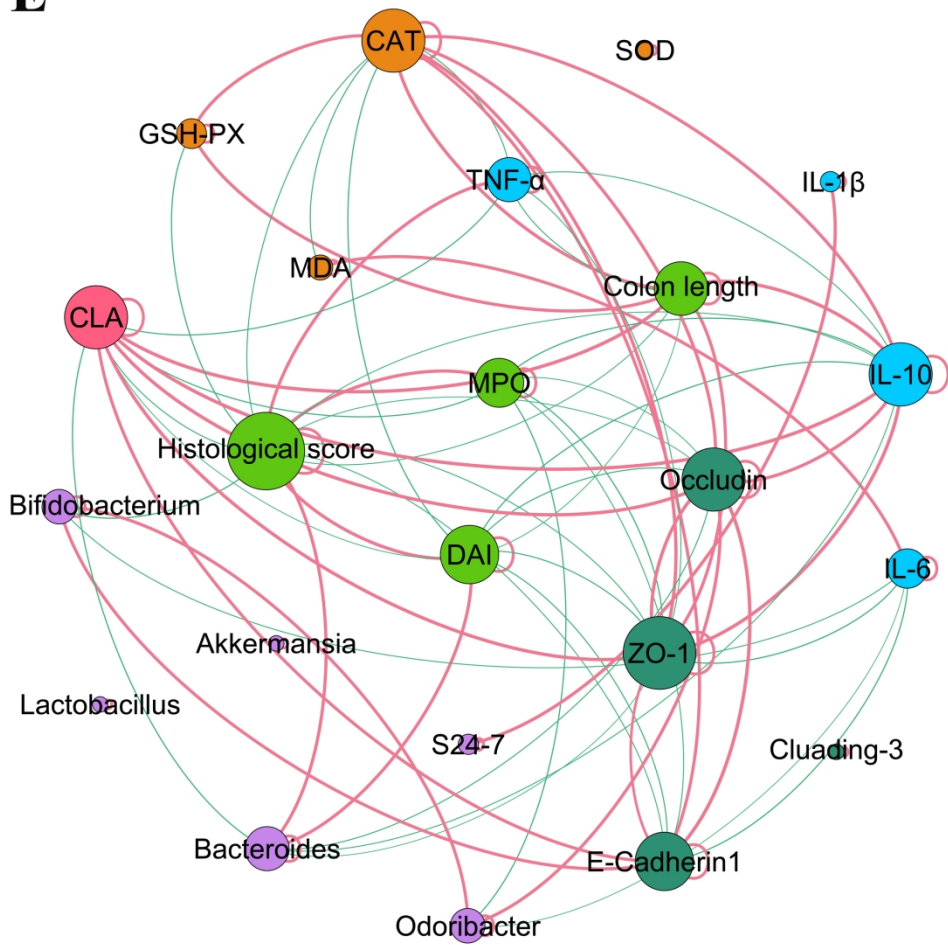
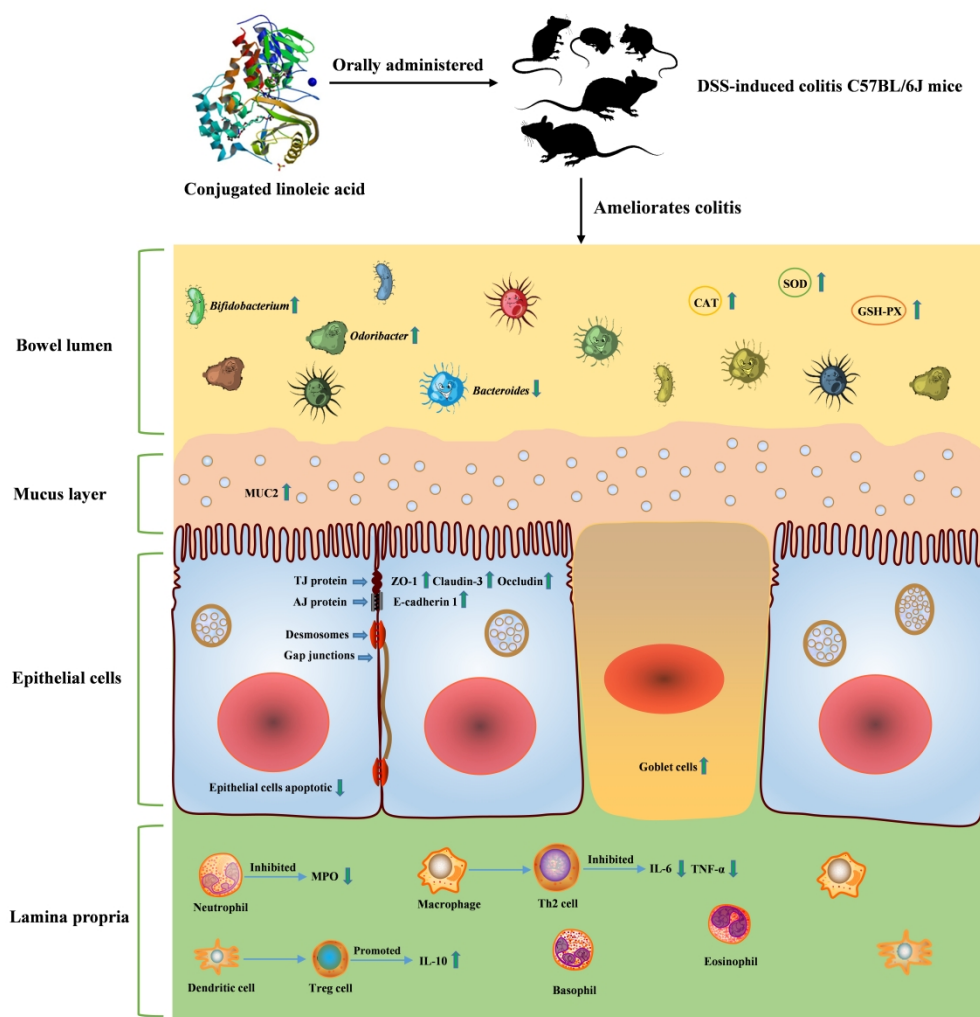


Fig.9E

209x213mm (300 x 300 DPI)



TOC Graphic The diagram illustrated the ways that CLA alleviates DSS-induced colitis in mice from four aspects: intestinal microorganisms, oxidation stress, intestinal barrier, inflammatory cytokines. 40 mg/d, 20 mg/d and 10 mg/d CLA significantly increased the concentration of MUC2 and goblet cells. Meanwhile, 40 mg/d CLA and 20 mg/d CLA treatments significantly up-regulated the concentration of tight junction proteins (ZO-1, occludin and claudin-3) and ameliorated epithelial apoptosis caused by DSS. Moreover, oxidative stress-related enzymes (SOD, GSH-PX, CAT) and inflammatory cytokines (TNF- $\alpha$ , IL-10, IL-6) were modulated by 40 mg/d CLA and 20 mg/d CLA. Furthermore, 40 mg/d CLA rebalanced the gut microbiota damaged by DSS, including reducing *Bacteroides* and increasing *Bifidobacterium* and *Odoribacter*.